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(57) Abstract

A method is described for inducing male sterility in plants which are capable of being genetically transformed. The plant is tranformed with a DNA-construct that combines a male-organ specific promoter with an enzyme which reacts with a protoxin to release, in the male-organ or gamete, a toxin for the male-organ or gamete. Also described is the DNA-construct, the protoxin, the transgenic plant containing the construct, seeds produced by the transgenic plant, a method for producing hybrid seed, seed progeny, and transgenic plants contacted with the protoxin.

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TITLE

COMPOUNDS AND CONSTRUCTS FOR PRODUCING MALE STERILE PLANTS

BACKGROUND OF THE INVENTION

Hybrid seed production is an important means of introducing desirable traits into agronomically valuable crop plants. For instance, quality traits such as oil content, herbicide resistance, disease resistance, adaptability to environmental conditions, and the like, can be hybridized in offspring so that the latter are invested with the most desirable traits of its parents. In addition, progeny from a cross may possess new qualities resulting from the combination of the two parental types, such as yield enhancement resulting from the phenomenon known as heterosis. Controlled cross-fertilization to produce hybrid seeds has been difficult to achieve commercially due to competing self-fertilization, which occurs in most crop plants.

Currently, hybrid seed production is performed by one of the following means: a) mechanically removing or covering the male organs or gametes to prevent self-fertilization followed by exposing the male-disabled plants to plants with fertile male organs that contain the trait(s) desired for crossing; b) growing genetically male-sterile plants in the presence of plants with fertile male organs that contain the trait that is desired for crossing; or c) treating plants with chemical hybridizing agents (CHA) that selectively sterilize male organs followed by exposing the male-disabled plants to plants with fertile male organs that contain the trait that is desired for crossing.

Some disadvantages to each of these methods include: a) this is only practical for a few crops, such as corn, where the male and female organs are

structurally far apart; and it is labor intensive and costly; b) genetically male sterile lines are cumbersome to maintain, requiring crosses with restorer lines; c) all CHAs exhibit some degree of general phytotoxicity and female fertility reduction. Also, CHAs often show different degrees of effectiveness toward different crop species, or even toward different varieties within the same species. The method of this invention is applicable to a wide range of crops and allows selfing to maintain lines.

This invention concerns, inter alia, a method for selectively sterilizing male organs in one parent and exposing the resulting plant to plants with fertile male organs to produce seed having the desirable characteristics of both parents and potentially additional characteristics resulting from the combination. Plants are rendered receptive to male sterility induction by introducing certain DNA constructs. Some background teachings concerning DNA constructs employed herein or related to plant male sterility are as follows.

EPA 89-329308 discloses constructs containing antisense DNA and other genes in tobacco, tomato and Brassica with the prediction that expression of the antisense RNA and toxic proteins will result in male sterility. Pollen-specific promoters and chemically regulated promoters are suggested. Also disclosed is the introduction of β-glucuronidase (GUS) into the transgenic plant as a marker gene but without recognition that GUS itself has utility in producing male sterility when the plant is exposed to glucuronide conjugates of toxins.

A note by Jefferson in Nature 342:838 (1989) describes that cytotoxic aglycones can be conjugated and

used to produc tissue-specific toxicity to GUS-expressing plant cells.

GB 2197653A discloses the production of transgenic plants containing GUS and the use of constructs to produce said plants. GUS is said to have utility as a reporter gene for promoter analysis and possibly as a means to select mutations in fused promoters.

EPA 89-344029 discloses the production of toxic proteins in the male organs of plants to produce male sterility. Also disclosed is the expression of GUS as a marker gene in male organs but without the recognition that GUS itself has utility in producing male sterility when the plant is exposed to glucuronide conjugates of toxins as disclosed herein.

An article by Goldberg in Science 240:1460-1467 (1988), discloses the anther-specific promoter from the tobacco TA29 gene. See also EPA 89-344029. This promoter is useful as a component of constructs employed in the instant method for producing male sterility in plants.

WO 90/08828 discloses that GUS cleaves a glyphosate-glucuronide protoxin into a glyphosate toxin. No teaching is given for the synthesis of glyphosate-glucuronide, no protoxin is synthesized, no demonstration is provided that glyphosate-glucuronide is a suitable substrate for GUS or that glyphosate-glucuronide is nontoxic in plants and there is no disclosure of sulfonylurea or maleic hydrazide toxins.

Each of the following publications describes representative sulfonylureas that can be conjugated to saccharides such as glucuronic acid according to the teachings herein and employed in the disclosed method for effecting male sterility in plants:

U.S. 4,394,506 U.S. 4,394,506

U.S. 4,481,029 EPA 87,780

EP 95,925	EPA 161,211
U.S. 4,435,206	Japan 63/166,803A
U.S. 4,522,645	U.S. 4,684,395
U.S. 4,420,325	EP 87,780A.

SUMMARY OF THE INVENTION

This invention concerns a method for inducing male sterility in economically important plants which are, or can be made, capable of genetic transformation. A plant of choice is transformed with a gene containing the coding region of an exogenous enzyme and a suitable male organ-specific promoter. The resulting transgenic plant produces the exogenous enzyme only in its male organ(s). Such transgenic plants are male-fertile when grown normally. Unlike normal plants, however, the plants of the invention can be rendered male-sterile by exposure to a protoxin that is converted to an active toxin by the exogenous enzyme in the male organ. The male sterility trait is only expressed when wanted, by contacting the plant with a selected protoxin; otherwise the transgenic plant behaves normally.

This "silent" male sterility characteristic is advantageous in many aspects of hybrid seed production. Since the protein product coded by the engineered gene is an exogenous enzyme (preferably, GUS) which by itself does not cause male sterility in the host plant, the gene can be easily carried in any sexual propagation or hybridization schemes. The male sterility trait is only "switched on", at a desired time, by contacting the plant with a protoxin. When such a transgenic plant is chosen as the female parent for large scale hybrid seed production, large quantities of its seed can be produced by selfing. An advantage of this invention is that the gene coding for the exogenous enzyme in the male organ can be introduced into any desirable breeding lines.

The line harboring the gene can be used either as the female or male parent.

More particularly, this invention pertains to an improved method for inducing male sterility in a plant having a male organ comprising the steps:

(i) transforming the plant with a DNA construct that combines a male-organ specific promoter with the coding region for β-glucuronidase; and

(ii) contacting the transformed plant with a glucuronic acid-containing protoxin to release a toxin which renders the male organ sterile;

wherein the improvement comprises:

(a) employing, in step (i), a promoter selected from the group $TA29_{1500}$, $TA29_{500}$ (SEQ ID NO:1), p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7); and

(b) employing, in step (ii), a protoxin that comprises a toxin conjugated through a non-acyl, non-phosphoryl hydroxyl residue to glucuronic acid.

Preferred is the method in which the plants are these genetically transformable agricultural and horticultural species: corn, rice, orchardgrass, soybean, cotton, Brassica (B. napus, B. campesteris, B. olerica), potato, sugarbeet, pea (Pisum sativum) alfalfa, sunflower, tobacco, flax, tomato, lettuce, celery, carrot, eggplant, pepper (Capsicum annuum) apple, melon, petunia, periwinkle, poplar and walnut.

Further, the male organ-specific promoter is the $TA29_{1500}$ promoter, $TA29_{500}$ promoter (SEQ ID NO:1), or promoters from Brassica anther-specific genes represented by the cDNA clones p73, p112, p54, p42W, p42, and p158 (SEQ ID NOS:2-7); the male organ is the anther; the exogenous enzyme is β-glucuronidase (GUS); and the protoxin is conjugated through a hydroxyl

residue to glucuronic acid. One skilled in the art, given the disclosure herein, will be able to isolate the promoters from the genes represent d by SEQ ID NOS:2-7 without undue experimentation.

This invention also pertains to protoxins that are formed from herbicides, chemical hybridizing agents and male sterilants linked to saccharides -- whose conjugates are cleaved slowly or not at all in higher plants -- via an oxygen atom. For simplicity hereafter, the term "herbicide" (includes "herbicide derivative") will be used to encompass herbicides, hybridizing agents and sterilants. Preferred protoxins are herbicidal sulfonylureas and maleic hydrazide linked to β-D-glucuronic acid.

Contemplated protoxins are described hereafter. It should be understood that glucuronic acid esters of these protoxins are included within the scope of this invention and that the term "protoxin" includes the following compounds and their glucuronic acid esters. In fact, an interesting aspect of this invention is the use of the ester form of the protoxin, solely or in various combinations with the free acid form, to induce male sterility over a pre-targeted period of time during which the male organ is known to develop. Extensive plant esterase activity will release the free acid glucuronide (or other non-ester form of the herbicide) in a time-controlled manner to eliminate the need for more than one application of protoxin even for plants in which male fertility would otherwise normally develop over several weeks.

The protoxins of this invention are: (i) derivatives of herbicidal sulfonylureas having a glucuronic acid group added to any methyl, methylene or methine carbon of the sulfonylurea, and (ii) glucuronides of chemical hybridizing agents (CHA) such

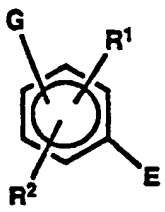
as maleic hydrazide. The protoxin is a water-stable compound in which the glucuronide is tether d with a 0 to 6, preferably 0 to 3, atom chain wh rein the chain is made up of carbon atoms or, if the tether is 2 or more atoms, one or more can be a sulfur, nitrogen or oxygen atom.

A number of hydroxyl-containing sulfonylureas are disclosed in the patents referred to in the "Background" (supra). Additionally, hydroxyl-containing sulfonylureas can be prepared by one skilled in the art from the other sulfonylureas disclosed in said patents.

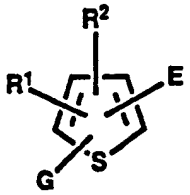
This invention employs sulfonylureas of Formula I, including their agriculturally suitable isomers, salts and derivatives thereof:

I

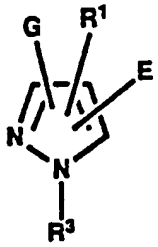
wherein J is



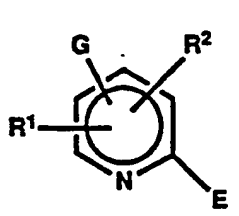
J-1



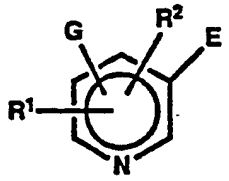
J-2



J-3



J-4



J-5

G is H or gluc-O(alkyl)$_n$L;

n is 0 or 1;

alkyl is 1 to 3 carbon atoms optionally substituted with one or two groups selected from halogen, methyl, methoxy or methylthio;

L is O, S(O)$_m$, NR^5, SO$_2$NR^4, CO$_2$, CH$_2$O, or a direct bond;

m is 0-3;

W is O or S;

R, R^4 and R^5 are independently H or CH$_3$;

E is a single bond or CH$_2$;

R^1 is H, C$_1$ to C$_3$ alkyl, C$_1$ to C$_3$ haloalkyl, halogen, nitro, C$_1$ to C$_3$ alkoxy, SO$_2$NR^{a}R^b, CONR^{a}R^b, C$_1$ to C$_3$ alkylthio, C$_1$ to C$_3$ alkylsulfinyl, C$_1$ to C$_3$ alkylsulfonyl, CH$_2$CN, CN, CO$_2$R^c, C$_1$ to C$_3$ haloalkoxy, C$_1$ to C$_3$ haloalkylthio, C$_2$ to C$_4$ alkoxyalkyl, C$_3$ to C$_4$ alkoxyalkoxy, C$_2$ to C$_4$ alkylthioalkyl, CH$_2$N$_3$, NR^{d}R^e, or Q;

R^2 is H, C$_1$ to C$_3$ alkyl, C$_1$ to C$_3$ haloalkyl, halogen, nitro, C$_1$ to C$_3$ alkoxy, C$_1$ to C$_3$ alkylthio, CN, C$_1$ to C$_3$ haloalkoxy, or C$_2$ to C$_4$ alkoxyalkyl;

R^a is H, C$_1$ to C$_4$ alkyl, C$_2$ to C$_3$ cyanoalkyl, methoxy or ethoxy;

R^b is H, C$_1$ to C$_4$ alkyl or C$_3$ to C$_4$ alkenyl; or

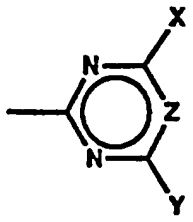
R^a and R^b can be taken together as $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$ or $-CH_2CH_2OCH_2CH_2-$;

R^c is C_1 to C_4 alkyl, C_3 to C_4 alkenyl, C_3 to C_4 alkynyl, C_2 to C_4 haloalkyl, C_2 to C_3 cyanoalkyl, C_5 to C_6 cycloalkyl, C_4 to C_7 cycloalkylalkyl or C_2 to C_4 alkoxyalkyl;

R^d and R^e are independently H or C_1 to C_2 alkyl;

Q is a saturated or partially saturated 5- or 6-membered carbocyclic ring, containing either one or two carbonyl groups, or a saturated or unsaturated 5- or 6-membered heterocyclic ring, containing 1 to 5 atoms of carbon and 1 to 4 heteroatoms selected from the group consisting of 0 to 2 oxygen, 0 to 2 sulfur and 0 to 4 nitrogen, wherein sulfur can take the form of S, SO or SO_2, and containing 0 to 2 carbonyl groups; Q can further be optionally substituted with 1 to 2 substituent groups; substituents on carbon can be selected from the group consisting of halogen, C_1 to C_4 alkyl, C_1 to C_4 haloalkyl, CH_2(C_2 to C_3 alkenyl), CH_2(C_2 to C_3 alkynyl), C_2 to C_4 alkoxycarbonyl, CN, OH, C_1 to C_3 alkoxy, C_1 to C_3 alkylthio, C_1 to C_3 alkylsulfinyl, C_1 to C_3 alkylsulfonyl or C_2 to C_4 alkylcarbonyl; substituents on nitrogen can be selected from the group consisting of C_1 to C_4 alkyl, C_1 to C_4 haloalkyl, CH_2(C_2 to C_3 alkenyl), CH_2(C_2 to C_3 alkynyl), C_2 to C_4 alkoxycarbonyl or C_2 to C_4 alkylcarbonyl;

A is



X is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkoxy, C_1 to C_4 haloalkyl, halogen, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to C_3 alkylamino, di(C_1 to C_3 alkyl)amino, C_3 to C_5 cycloalkyl, C_1 to C_4 alkyl substituted with -O-gluc, C_2 to C_4 alkoxyalkyl substituted with -O-gluc, or C_1 to C_4 alkoxy substituted with -O-gluc;

Y is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkoxy, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to C_3 alkylamino or di(C_1 to C_3 alkyl)amino;

R^3 is H or C_1 to C_3 alkyl;

Z is CH or N;

E^1 is a direct bond or CH_2;

gluc is β-D-glucuronic acid;

provided that:

(i) when G is H then X is C_1 to C_4 alkyl substituted with gluc, or C_1 to C_4 alkoxy substituted with gluc;

(ii) when L is not a direct bond, then n is not zero and alkyl comprises at least two carbon atoms which can be substituted as described.

Preferred are compounds of Formula I wherein

W is O;

R^1 is H, halogen, C_1 to C_3 haloalkoxy, $SO_2NR^aR^b$ $CONR^aR^b$, CO_2R^c, C_3-C_4 alkoxyalkoxy or Q;

R^2 is H, methyl, halogen, methoxy or trifluoromethyl;

R^a and R^b are, independently, H or C_1 to C_4 alkyl, or R^a and R^b can be taken together as $-(CH_2)_4-$, $-(CH_2)_5-$ or $-CH_2CH_2OCH_2CH_2-$;

R^c is C_1 to C_4 alkyl, C_2 to C_4 haloalkyl or C_2 to C_4 alkoxyalkyl;

X is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkyl, C_1 to C_4 haloalkoxy, halogen, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, C_1 to C_3 alkylamino, C_1 to C_4 alkyl substituted with -O-gluc, C_2 to C_4 alkoxyalkyl substituted with -O-gluc or C_1 to C_4 alkoxy substituted with -O-gluc.

More preferred are compounds of Formula I wherein:

R^1 is H, halogen, $SO_2NR^aR^b$, $CONR^aR^b$, CO_2R^c or tetrazolyl;

J is J-1, J-2, J-3 or J-4;

G is H, gluc-O- or gluc-O-(alkyl)L;

L is O, $S(O)_m$, CO_2, NR^5 or a direct bond; and m is 0 or 2.

Still more preferred are compounds of Formula I wherein:

R^1 is H, Cl, $CONR^aR^b$ or CO_2R^c;

J is J-1 or J-4;

G is H, gluc-O- or gluc-O-CH_2CH_2-L

L is O, S, SO_2, NR^5 or CO_2;

E is a single bond;

R^a and R^b are, independently, H or C_1 to C_4 alkyl;

R^c is C_1 to C_4 alkyl;

X is H, C_1 to C_3 alkyl, C_1 to C_3 alkoxy, Cl, C_1 to C_3 alkylamino or C_1 to C_3 alkyl substituted with -O-gluc, C_2 to C_3 alkoxyalkyl substituted with -O-gluc; or C_1 to C_3 alkoxy substituted with -O-gluc; and

Y is C_1 to C_3 alkyl, C_1 to C_3 alkoxy, C_1 to C_3 haloalkoxy, C_1 to C_3 alkylamino or C_1 to C_3 alkyl.

Most preferred are the glucuronides of N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-6-[(2-hydroxyethyl)methylamino]-2-pyridine-sulfonamide; and 2-[(2-hydroxyethyl)thio]N-[[(4-methoxy-6-methyl-2-pyrimidinyl)amino]-carbonyl]benzenesulfonamide.

This invention also pertains to the recited plants containing a novel DNA construct comprising a male organ-specific promoter operably linked upstream from GUS. Preferred are transgenic plants containing the $TA29_{1500}$ or $TA29_{500}$ promoter linked to GUS. This invention also pertains to the seeds produced by the transgenic plants and all progeny that exhibit the desirable traits herein described. This invention also pertains to vectors containing a novel DNA construct comprising a particular male organ-specific promoter operably linked upstream from GUS. Preferred is the PZS96 Agrobacterium binary vector containing the NptII marker gene and the $TA29_{1500}$ or $TA29_{500}$ promoter linked to GUS. This invention also pertains to a method of hybrid seed production and to the transgenic plants that have been exposed to the glucuronide conjugates. Finally, this invention concerns the promoter $TA29_{500}$ characterized by its ability to reliably direct anther specific expression in transgenic plants. $TA29_{500}$ comprises a nucleic acid fragment derived from the TA29 gene that extends from the EcoRV restriction site, which is about 500 base pairs 5' to the transcription start site and extending to the translation initiation ATG.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the maps of Agrobacterium tumefaciens binary vectors containing the TA29/P-GUS-Nos 3' chimeric genes. A) pZ6ASG: The 500 bp EcoRV-NcoI TA29 promoter combined with the GUS coding region and the Nos 3' region as described in Example 1 is adjacent to the NosP-NptII-Ocs 3' selection marker gene. Both of these genes are between the T-DNA borders in the binary vector pZS96, which includes a gene for ampicillin resistance (Amp^R) and an origin of replication from pBR322 (ori), and the replication (rep pVS1) and stability (sta) regions from pVS1. B) pZ6ALG: As in A) except that the 1500 bp ClaI-NcoI TA29 promoter is combined with GUS-Nos 3' as described in Example 2.

Figure 2 shows the GUS enzyme activity assayed in anthers of transgenic plants containing the $TA29_{500}$/P-GUS-Nos 3' gene as described in Example 3. Figure 2A represents a wild type tobacco plant, which shows virtually no endogenous GUS activity. Figures 2B and 2C show the anther-specific enzyme activity in two independent transformants that demonstrate anther-specific expression of GUS. Substantially no activity was observed in wild-type or transformed plant ovules or leaves (old or new).

DETAILS OF THE INVENTION

Definitions

Terms that are used frequently throughout the description are defined as follows:

"Male sterility" of a plant refers to the inability of the plant to produce fertile, functional pollen to germinate on the stigma and to effect the fertilization of the egg.

"Male organ" of the plant refers to the part of the flower that physically contains pollen. Pollen grains, at all stages of development, are considered a part of

the male organ. In general, male organ denotes "stamen" which includes the filament, anther and pollen in the anther. For the sake of simplicity, the term is intended to include the "gamete" as well. By "gamete" is meant a mature germ cell capable of forming a new individual by fusion with another gamete.

"Tapetum" refers to the innermost layer of anther wall which surrounds the developing pollen. At a late stage of pollen maturation, the tapetum layer disintegrates entirely.

"DNA-construct" refers to a linear or circular molecule of deoxyribonucleic acid (DNA), that is a composition of DNA fragments derived from any source.

An "exogenous enzyme" refers to an enzyme produced according to the information in the coding region of a gene which is introduced by transformation into the plant.

A "chemical hybridizing agent" (CHA) means a chemical that can render a plant partially or fully male sterile when it is applied to the plant.

The term "protoxin" refers to a chemical that releases a toxin upon reaction with the exogenous enzyme. Without interacting with the exogenous enzyme, the protoxin can also be converted into the toxin under certain chemical conditions. An agent that is "toxic" to the male organ refers to an agent that kills or renders the male organ non-functional. It may cause the production of dead pollen, or living but non-functional pollen, or both. The living but non-functional pollen cannot effect egg fertilization when applied to the stigma of the pistil.

In the molecular sense, the term "hybridization" means the bonding of complementary segments of DNA to DNA or RNA to DNA.

The term "hybridization", in the biological sense, refers to the production of offspring by crossbreeding of two plants that ar genetically different.

The "female parent" in hybridization means the plant whose eggs in the ovary of the flower are fertilized by the pollen of the male parent.

"Beta-glucuronidase" (GUS) is any enzyme that catalyzes the hydrolysis of β-O-glucuronide conjugates into glucuronic acid and the aglycone. In particular, GUS catalyzes the hydrolysis of a glucuronide conjugate protoxin into the toxin and glucuronic acid.

The term "glucuronide" is used interchangeably with "beta-glucuronide". It denotes a derivative of glucuronic acid in which a compound (the aglycone) is conjugated through the beta linkage to the oxygen atom on carbon number 1 of the glucuronic acid.

The "aglycone" of the glucuronide refers to any compound that is conjugated to the glucuronic acid. The chemical nature of the aglycone is limited only by the requirement that it possess a conjugatable hydroxyl group.

The expression "nucleotide sequence" refers to a polymer of DNA or RNA, which can be single- or double-stranded, optionally containing synthetic, non-natural, or altered nucleotides capable of incorporation into DNA or RNA polymers.

"DNA segment" refers to a linear fragment of single- or double-stranded DNA derived from any source. The expression "DNA in plant cells" includes all DNA present in plant cells. As used herein, a "gene" is intended to mean a DNA segment which includes a 5' regulatory region, a coding region, and a 3' polyadenylation nucleotide region and is normally regarded as a gene by those skilled in the art.

"Coding region" refers to a DNA segment which encodes a regulatory molecule or any polypeptide.

"Gene product" refers to a polypeptide resulting from transcription, translation, and, optionally, post-translational processing of a selected DNA segment.

The term "expression" as used herein is intended to mean the translation to gene product from a gene coding for the sequence of the gene product. In expression, a DNA chain coding for the sequence of gene product is first transcribed to a complementary RNA which is called a messenger RNA and then, the thus transcribed messenger RNA is translated into the above-mentioned gene product.

The term "promoter" or "promoter region" refers to a sequence of DNA, usually upstream (5') of the coding sequence, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site. A "promoter fragment" constitutes a DNA sequence consisting of the promoter region. A promoter region can include one or more regions which control the effectiveness of transcription initiation in response to physiological conditions, and a transcription initiation sequence.

"Tissue specific promoters" are those that direct gene expression primarily in specific tissues.

A "male organ-specific promoter" refers to a promoter that directs gene expression primarily in the male organ, i.e., pollen, anther tissues, filament of the anther, and gamete. Transcription stimulators, enhancers or activators can be integrated into these promoters to create a promoter with a high level of activity that retains its specificity.

"Regulatory nucleotide sequence" refers to a nucleotide sequence located proximate to a coding region whose transcription is controlled by the regulatory

nucleotide sequence in conjunction with the gene xpression apparatus of th cell. Generally, th regulatory nucleotide s quence is located 5' to the coding region. A promoter can include one or more regulatory nucleotide sequences.

"Polyadenylation nucleotide sequence" (or "region") refers to a nucleotide sequence located 3' to a coding region which controls the addition of polyadenylic acid to the RNA transcribed from the coding region in conjunction with the gene expression apparatus of the cell.

"Transformation" means the process by which cells/tissues/plants acquire properties encoded on a nucleic acid molecule that has been transferred to the cell/tissue/plant. "Transferring" refers to methods to transfer DNA into cells including, but not limited to, microinjection, permeabilizing the cell membrane with various physical (e.g., electroporation) or chemical (e.g., polyethylene glycol, PEG) treatments, high-velocity microprojectile bombardment also termed biolistics, or infection with Agrobacterium tumefaciens or A. rhizogenes.

"Transformant" or "transgenic plant" means a plant which has acquired properties encoded on a nucleic acid molecule that has been transferred to cells during the process known as transformation.

"Integrated" means that the DNA is incorporated into the plant genome.

In the present invention, the β-glucuronidase enzyme is specifically produced in anther or pollen cells. GUS catalyzes the hydrolysis of a wide variety of glucuronides. Most any aglycone conjugated to D-glucuronic acid through a β-O-glycosidic linkage is a suitable substrate. GUS enzyme activity can be easily assayed using a number of methods including

spectrophotometrically using p-nitrophenyl-glucuronide as the substrate, fluorometrically using 4-methyl umbelliferyl-glucuronide (MUG) as th substrate, or histochemically using 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) as the substrate.

The longevity, broad pH optimum, broad substrate range, good heat tolerance, lack of co-factors, and particularly the absence of measurable amounts of the enzyme in higher plants combine to make β-glucuronidase a very desirable enzyme to generate a toxin from a glucuronide protoxin.

The GUS enzyme is naturally encoded by the uidA gene of Escherichia coli. The cloning and characterization of this gene, and subcloning of the coding region, is described by Jefferson et al., Proc. Natl. Acad. Sci. USA 83: 8447-8451 (1986). The use of this coding region in conjunction with various promoter regions, thereby providing the GUS enzyme as a marker for their expression, is well established in the art, for example, in Jefferson et al., EMBO J 6: 3901-3907 (1987), Benfey et al., EMBO J 8: 2195-2202 (1989), Broglie et al., The Plant Cell 1: 599-607 (1989), Keil et al., EMBO J 8: 1323- 1330 (1989), and Keller et al., EMBO J 8: 1309-1314 (1989). A chimeric gene composed of the GUS coding region and the CaMV 35S promoter is available from Clontech, Inc.

The GUS enzyme is expressed as the product of a chimeric gene that is transferred into plants, that includes a regulatory nucleotide sequence which directs expression specifically in cells located in the anther or in pollen. Suitable regulatory nucleotide sequences are known in the art. The particular anther or pollen-specific promoter which is employed with a selected plant species is not critical to the method of the invention. A partial list of suitable promoters

includes those from the TA29 and TA13 tobacco genes described by Goldberg, Science, 240: 1460-1467 (1988), the LAT52 tomato g ne d scribed by Twell et al, Mol. Gen. Genet. 217: 240-245 (1989), the Zmc13 and pZmc26 corn genes described by Stinson et al., Plant Physiol. 83: 442-447 (1987), the pTpc70 and pTpc44 Tradescantia genes also described by Stinson et al., and the CHI-A (P_{A2} region) and CHI-B petunia genes described by van Tunen et al., Plant Mol. Biol. 12: 539-551 (1989). Novel anther promoters are derived from Brassica napus genes corresponding to the newly-identified anther-specific cDNAs with SEQ ID NOS:2-7. Most preferably, the regulatory nucleotide sequence is a TA29 promoter.

Methods for introducing a DNA sequence into plant cells are known in the art. Nucleic acids can generally be introduced into plant protoplasts, with or without the aid of electroporation, polyethylene glycol, or other processes known to alter membrane permeability. Nucleic acid constructs can also be introduced into plants using vectors comprising part of the Ti- or Ri-plasmid, a plant virus, or an autonomously replicating sequence. Nucleic acid constructs can also be introduced into plants by microinjection or by high-velocity microprojectiles, also termed "particle bombardment" or "biolistics" (Sanford, Tibtech 6: 299 (1988)), directly into various plant parts. The preferred means of introducing a nucleic acid fragment into plant cells involves the use of Agrobacterium tumefaciens containing the nucleic acid fragment between T-DNA borders in a binary vector in trans to a disarmed Ti-plasmid. The Agrobacterium can be used to transform plants by inoculation of tissue explants, such as stems, roots, or leaf discs, or by co-cultivation with plant protoplasts.

The range of crop species in which foreign genes can b introduced is increasing rapidly as tissue culture and transformation methods improve and as appropriate selectable markers become available. Thus, this invention is applicable to a broad range of agronomically or horticulturally useful plants. The particular method which is employed to introduce the DNA sequence into a selected plant cell is not critical. In a preferred embodiment, DNA sequences are introduced into plant cells by co-cultivation of leaf disks or plant tissue explants with Agrobacterium tumefaciens.

Most preferably, the plasmid for introducing a DNA sequence comprising an anther or pollen specific promoter, a GUS coding region, and a nopaline synthase gene polyadenylation nucleotide sequence (NOS 3') is pZ6ASG or pZ6ALG or derivatives thereof. These plasmids can be used to generate plants that express the GUS enzyme in their anthers by those skilled in the art or as taught in this application.

In the present method, plants expressing GUS in the anthers or pollen are contacted with a protoxin, such as a general cytotoxic agent or a herbicide that has been conjugated through an oxygen atom to glucuronic acid. The protoxin is transported to all regions of the plant, but is efficiently cleaved to toxin only in the male organs and exhibits cytotoxicity only to the male organs. The plant is thus rendered male-sterile upon exposure to the protoxin. As will be appreciated by one skilled in the art, not all protoxins will be equally effective as male-sterilants in all plants that have anther-specific expression of GUS. Some of them may be toxic to certain plants. Others may have relatively low toxicity to the male organ. Nevertheless, given the disclosure presented herein, and with a minimum of experimentation, one skilled in the art will be able to

easily determine which protoxin(s) to employ with a specific plant.

EXAMPLES

Methods of culturing bacteria, preparing DNA, and manipulating DNA were as described by Maniatis et al., Molecular Cloning: A Laboratory Manual [Cold Spring Harbor Laboratory, New York (1982)] unless stated otherwise. Restriction enzymes and other enzymes used in DNA manipulations were obtained from New England Biolabs, Inc. (Beverly, MA, USA), Boehringer Mannheim (Indianapolis, IN, USA), or Bethesda Research Laboratory (Gaithersburg, MD, USA) and were used according to the manufacturer's specifications.

EXAMPLE 1

A chimeric gene was constructed to obtain expression of the β-glucuronidase (GUS) coding region in the tapetal cells of anthers. The promoter was derived from the tobacco TA29 gene, a gene that is expressed naturally only in the tapetal tissue of the tobacco anther. A clone containing the TA29 gene was obtained from Dr. Goldberg at the University of California, Los Angeles. The TA29 gene can also be obtained by methods taught in EPA 84-344,029. One skilled in the art can prepare a probe to the TA29 cDNA sequence given in Figure 2 of '029 and isolate a TA29 gene-containing clone from a tobacco genomic library using that probe. The TA29 gene sequence is given in Fig. 3 of EPA 89-344029.

A TA29 promoter fragment was prepared from the TA29 gene by first cloning an SstI-HindIII fragment, which was expected to contain the TA29 promoter region based on the location of the 5' end of the messenger RNA, from the Goldberg clone into the SstI and HindIII digested vector M13mp18. This fragment proved too large to carry out further steps so an approximately

500 base pair (bp) EcoRV-HindIII fragment was isolated and cloned into SmaI and HindIII digested M13mp19. (EcoRV and SmaI both leave blunt nds.) The translation initiation ATG was identified and the sequence of DNA surrounding it was determined by sequencing in from the HindIII end of the fragment according to the method of Sanger et al., Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977) using a U.S. Biochemical Corporation Sequenase DNA sequencing kit and following the manufacturer's protocol. Next, an oligonucleotide with the sequence AGAAATTAGCTACCATGGTAGCTCCAAAAT (SEQ ID NO:8) was synthesized using an Applied Biosystems DNA synthesizer and following the manufacturer's procedure. This oligonucleotide was used in a site-directed mutagenesis procedure as described in Viitanen et al., J. Biol. Chem., 263:15000-15007 (1988), to create an NcoI site surrounding the translation initiation ATG. The approximately 500 bp TA29 promoter fragment containing the new NcoI site was then moved as an SstI-HindIII fragment, the SstI site being derived from the M13mp19 polylinker, into SstI and HindIII digested pTZ19 (available from Pharmacia) creating pTZAS. Next, an NcoI-HindIII fragment that includes the GUS coding region and a nopaline synthase gene polyadenylation nucleotide sequence (NOS 3') was prepared from the plasmid pTZCGNC.

The pTZCGNC vector contains pTZ19 and a chimeric NOS/P-GUS-NOS 3' gene and was constructed in the following manner. A GUS coding region fragment was prepared from pRAJ275, which is described in Jefferson et al., Proc. Natl. Acad. Sci. USA 83, 8447-8451 (1986) and is available from Clontech Laboratories. The plasmid, pRAJ275, was digested with EcoRI, the end made blunt, and a BamHI linker was added. It was then

digested with HindIII to prepare a HindIII-BamHI fragment containing the GUS coding region. This fragment was ligated into HindIII and BamHI digested pKNK creating pKNGUS. The plasmid, pKNK, bears ATCC deposit accession number 67284. It is a pBR322 based vector which contains a neomycin phosphotransferase II (NptII) promoter fragment, a nopaline synthase (NOS) promoter fragment, the coding region of NptII and the polyadenylation nucleotide sequence from the NOS gene. A map of this plasmid is shown in Lin et al., Plant Physiol. 84: 856-861 (1987). The 320 bp ClaI-BglII fragment in pKNK that contains the NptII promoter was obtained as a HindIII-BglII fragment from the NptII gene of the transposon Tn5 described by Beck et al., Gene 19: 327-336 (1982). The HindIII site was converted to a ClaI site by linker addition. The NptII promoter fragment is followed by a 296 bp Sau3A-PstI NOS promoter (NOS/P) fragment corresponding to nucleotides -263 to +33, with respect to the transcription start site, of the NOS gene described by Depicker et al., J. Appl. Genet. 1: 561-574 (1982). The PstI site at the 3' end was created at the translation initiation codon of the NOS gene. The NOS/P is followed by a 998 bp HindIII-BamHI sequence containing the NptII coding region obtained from the transposon Tn5 [Beck et al., Gene 19: 327-336 (1982)] by the creation of HindIII and BamHI sites at nucleotides 1540 and 2518, respectively. The NptII coding region is then followed by a 702 bp BamHI-ClaI fragment containing the 3' end of the nopaline synthase gene including nucleotides 848 to 1550 [Depicker et al., J. Appl. Genet. 1: 561-574 (1982)]. The remainder of pKNK consists of pBR322 sequences from 29 to 4361.

From pKNGUS, a ClaI fragment containing the chimeric NOS/P-GUS-NOS 3' gene was isolated, the ends

were made blunt, and it was ligated into the SmaI site of pTZ19 creating pTZCGNC. An NcoI-HindIII fragment containing the GUS coding r gion and NOS 3' was isolated, the HindIII site being derived from the pTZ polylinker, and it was ligated into NcoI and HindIII digested pTZAS creating the chimeric $TA29_{500}$/P-GUS-NOS 3' gene in the plasmid pTZASG. The resulting plasmid contains a chimeric gene that has a 500 bp TA29 promoter and a NOS 3' as the regulatory signals surrounding the GUS coding region in the vector pTZ19.

This chimeric gene was transferred into pZS96, a binary vector used in Agrobacterium tumefaciens transformations of plant tissue. The plasmid, pZS96, contains the origin of replication and ampicillin resistance gene from pBR322 for maintenance and selection in E. coli. It contains the replication and stability regions of the Pseudomonas aeruginosa plasmid pVS1, described by Itoh et al., Plasmid 11: 206-220 (1984), which are required for replication and maintenance of the plasmid in Agrobacterium. Also contained are a T-DNA left border fragment of the octopine Ti plasmid pTiA6 and a right border fragment derived from TiAch5 described by van den Elzen et al., Plant Molec. Biol. 5: 149-154 (1985). Between these borders are a NOS/P-NptII-OCS 3' chimeric gene to confer kanamycin resistance to plant cells and a LacZ gene with the unique restriction sites HindIII, SalI, BamHI, SmaI, KpnI, and EcoRI derived from pUC18. pZS96 was digested with SmaI and HindIII and ligated to a ScaI-HindIII fragment containing the $TA29_{500}$/P-GUS-NOS 3' gene isolated from pTZAS, the ScaI site being located in the pTZ sequence. (ScaI and SmaI digests both leave blunt ends.) The resulting plasmid called pZ6ASG (Figure 1A) contains a chimeric gene that has a 549 bp TA29 promoter (SEQ ID NO:1) and a NOS 3' as the

regulatory signals surrounding the GUS coding region in the vector pZS96.

EXAMPLE 2

A second promoter fragment from the TA29 gene was prepared by first isolating an approximately 1500 bp ClaI-HindIII fragment from the SstI-HindIII fragment that was subcloned from the Goldberg TA29 gene clone described above. During the isolation, the ClaI end was filled in so that this fragment could be cloned into the HincII (blunt end) and HindIII sites of M13mp19. The same method described above was used to create an NcoI site surrounding the translation initiation ATG. The TA29 promoter fragment containing the new NcoI site was then moved as a SmaI-HindIII fragment, the SmaI site being derived from the M13mp19 polylinker, into SmaI and HindIII digested pTZ19 creating pTZAL. Before adding the GUS-NOS 3' fragment adjacent to the promoter, the ClaI fragment containing the NOS/P-GUS-NOS 3' gene in pTZCGNC was isolated, the ends made blunt, and it was cloned into SphI digested and blunted pTZ19. This step was carried out to eliminate the polylinker sites that are located between the NOS 3' and the HindIII site in pTZASG. The NcoI-HindIII fragment that includes the GUS-NOS 3' was prepared from the resulting plasmid and it was cloned into NcoI and HindIII digested pTZAL creating pTZALG. An Asp718-HindIII fragment containing the entire chimeric $TA29_{1500}$/P-GUS-NOS 3' gene was ligated into Asp718 and HindIII digested pZS96, described above, creating pZ6ALG (Figure 1B). The resulting plasmid is unique in that it contains a chimeric gene that has with the 1500 bp TA29 promoter and GUS coding region, a NOS 3' as the polyadenylation regulatory signal, and this chimeric gene is present in the pZS96 vector.

EXAMPLE 3

The chimeric $TA29_{500}$/P-GUS-NOS 3' gene, described above, was introduced into tobacco by Agrobacterium tumefaciens infecti n of leaf disks. Primary transformants were analyzed to demonstrate anther specific expression of this gene.

The plasmid pZ6ASG was transferred into Agrobacterium tumefaciens by a method involving a three-way mating that was essentially as described by Fraley et al., Proc. Natl. Acad. Sci. USA, 80: 4803-4807 (1983) except for the following points. The plasmid, pZ6ASG, was mated into Agrobacterium strain LBA4404 described by Hoekema et al., Nature 303: 179-180 (1983). Colonies from the pZ6ASG mating were selected on MinA (Table 1) plates containing 100 µg/mL rifampicin, 25 µg/mL carbanicillin, and 10 µg/mL tetracycline. Selected colonies were confirmed as transformants by restriction digests of miniprep DNA. To prepare a culture for plant transformation, 5 mL of MinA liquid medium (without agar) was inoculated with a single Agrobacterium colony harboring pZ6ASG and grown for approximately 17-20 hours in 18 mm glass culture tubes in a New Brunswick platform shaker maintained at 28°C.

Standard aseptic techniques for the manipulation of sterile media and axenic plant/bacterial cultures were followed, including the use of a laminar flow hood for all transfers. Potted tobacco plants for leaf disk infections were grown in a growth chamber maintained for a 14 hr, 21°C day, 10 hr, 18°C night cycle, with approximately 80% relative humidity, under mixed cool white fluorescent and incandescent lights. Tobacco leaf disk infection was carried out essentially by the method of Horsch et al., Science 227: 1229-1231 (1985), omitting nurse cultures, as described below.

Healthy young leaves, not fully expanded and approximat ly 4-5 inches in length, were harv sted from approximately 5-7 week old tobacco plants (Nicotiana tabacum var. Xanthi). The leaves were surface sterilized for 30 minutes by immersion in a solution containing 10% Clorox® and 0.1% sodium dodecyl sulfate (SDS) and then rinsed three times with sterile deionized water. Leaf disks, 8 mm in diameter, were prepared from whole leaves using a sterile paper punch. Leaf disks were inoculated by submerging them for several minutes in 20-30 mL of a 1:10 dilution of the overnight culture of Agrobacterium harboring the pZ9ASG plasmid. After inoculation, the leaf disks were placed on CN agar medium (Table 1) in petri dishes which were then sealed with parafilm. The petri dishes were incubated under mixed fluorescent and grow lights for 2-3 days in a culture room maintained at approximately 25°C.

To rid the leaf disks of Agrobacterium and to select for the growth of transformed tobacco cells, the leaf disks were transferred to fresh CN agar medium containing 500 mg/L cefotaxime and 100 mg/L kanamycin. Leaf disks were incubated under the growth conditions described above for 3 weeks and then transferred to fresh media of the same composition.

Approximately 2 weeks later, shoots developing on kanamycin-containing medium were excised with a sterile scalpel and planted in Root Induction medium 'A' (Table 1) containing 500 mg/L cefotaxime and 100 mg/L kanamycin. Root formation was recorded within 3 weeks. Rooted shoots were then transplanted to wet Metro-Mix in 8-inch pots, moved to a growth chamber set at the conditions described above, and covered with plastic bags for 7-10 days. After 4-6 weeks, plants were

transferred to the greenhouse and allowed to grow to maturity.

To assess th anther-specific expression of the introduced chimeric $TA29_{500}$/P-GUS-NOS 3' gene different plant tissues were tested for GUS enzyme activity as follows. For each plant, protein extracts were made from the collected five anthers of a young flower bud of stage 3-4 [the time of peak TA29 expression according to Goldberg, Science 240: 1460-1467 (1988)], from the ovule and pistil, from 0.05 g of tissue from a young leaf, and from 0.05 g of tissue from an old leaf. Each tissue sample was ground on ice in a microfuge tube containing 125-200 µL of GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% triton X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol) with a Kimble pellet pestle. The extracts were centrifuged in a microfuge for 5 minutes, and the supernatants transferred to new tubes. Then, 150 µL of each supernatant (with more GUS extraction buffer added if necessary) was transferred to another tube, 16.7 µL of a 10 mM 4-methyl umbelliferyl-glucuronide (MUG) solution in GUS extraction buffer was added as the GUS enzyme substrate, mixed, and 30 or 40 µL samples were transferred to a microtitre plate as time point samples at 0, 2, and 4 hours or at 10, 20, 30, and 60 minute intervals. Each well in the microtitre plate was previously filled with 360 µL of 0.2 M sodium carbonate to stop the reaction. Fluorescence of the reaction product, 4MU, was measured using a Perkin-Elmer plate reader with the excitation setting at 365 nm and the reading taken at 455 nm. The protein concentration of each supernatant extract was determined using the BioRad Protein Assay reagent according the the manufacturer's protocol. The data was compiled as

nanomoles of 4MU produced per mg of protein at each time point.

Sixteen independent tobacco transformants were assayed as described above with the following results. 56% of the plants (9/16) showed tightly regulated anther specific expression of the $TA29_{500}$/P-GUS-NOS 3' gene in that the ovule, new leaf, and old leaf samples had essentially no GUS activity, as seen in the wild type control tobacco plant samples, and substantial GUS activity was present in the anther sample (Figure 2). Twenty-five percent of the plants (4/16) showed substantial GUS activity in the anther sample, but the level in one or more of the other tissues tested was greater that that in the control. This category of transformant was classified as having anther specific expression with high background. One plant (6%) had non-specific activity in that the anther GUS activity was lower than in other samples. Finally, 13% of the plants (2/16) had no GUS activity. These results indicate that though not every transformant exhibits the desired pattern of $TA29_{500}$/P-GUS-NOS 3' expression, the 500 bp TA29 promoter fragment is capable of directing anther specific expression of GUS in individual transformed tobacco plants.

To verify that anthers of buds at stages 3 and 4 show the peak $TA29_{500}$/P-GUS-NOS 3' expression levels, a time course of GUS activity was established in anthers from two plants exhibiting anther specific GUS expression. Anthers from buds of stages 2,3,4, and 6 were assayed for GUS enzyme activity using the fluorescent assay described above. In each case the activity was highest at stage 3.

Seeds were collected following self fertilization of individual transformants. To determine the number of genetic loci for the $TA29_{500}$/P-GUS-NOS 3' gene, and

linked kanamycin resistance marker, s eds were germinated on plat s of Root Induction medium 'A' containing 100 or 200 µg/mL kanamycin. S eds wer sterilized by incubation for 30 minutes in 10% Clorox® and 0.1% SDS and sewn at 60 seeds per plate. Sensitive seeds germinate, but the seedlings bleach after 2-3 weeks. A segregation ratio of 3 resistant to 1 sensitive indicates the presence of a single site of integration of the kanamycin resistance gene into the genome of the transformant, which is then stably inherited by its progeny. This was seen in 3 out of 6 of the transformants with anther specific GUS expression that were tested. The remaining transformants exhibited a ratio which was greater than 3:1, indicating the presence of more than one integration site.

To establish lines of plants homozygous for the $TA29_{500}$/P-GUS-NOS 3' gene, 4-7 kanamycin resistant plants from lines showing 3:1 segregation in the seed germination test were potted in soil and grown to maturity in the greenhouse. Seed were collected after self fertilization and germinated on kanamycin medium as described above. Populations of seed that were 100% kanamycin resistant representing homozygous lines were identified.

TABLE 1

MinA

Per 500 mL:	100 mL: 5.25 g K_2HPO_4, 2.25 g KH_2PO_4, 0.5 g $(NH_4)_2 \cdot SO_4$, 0.25 g sodium citrate.$2H_2O$
	0.5 mL of 0.2 g/mL $MgSO_4 \cdot 7H_2O$
	5 mL of 20% sucrose or glucose
	7.5 g agar

Leaf Shoot Medium 'CN'

Per liter: 1 pkg MS Minimal Organic Medium with sucrose (Gibco)
1 mL of 1 mg/mL benzylaminopurine (BAP)
pH 5.6
8 g agar

Root Induction Medium 'A'

Per liter: 1 pkg MS Minimal Organic Medium without sucrose (Gibco)
10 g sucrose
pH 5.6
8 g agar

All of the above can be autoclaved after adding hormones.

EXAMPLE 4

The chimeric $TA29_{1500}$/P-GUS-NOS 3' gene was introduced into tobacco by Agrobacterium tumefaciens infection of leaf disks as described above using Agrobacterium harboring the pZ6ALG plasmid. Primary transformants were analyzed to demonstrate anther specific expression of the introduced chimeric GUS gene using the enzyme fluorescence assay as described above.

The twenty-four independent tobacco transformants that were assayed were placed in the same categories described for the $TA29_{500}$/P-GUS-NOS 3' transformants. Forty-two percent of the plants (10/24) showed tightly regulated anther specific expression of the $TA29_{1500}$/P-GUS-NOS 3' gene; 17% of the plants (4/24) had anther specific expression with high background; 25% of the plants (6/24) had non-specific activity. Finally, 17% of the plants (4/24) had no GUS activity. These results indicate that though not every transformant exhibits the desired pattern of $TA29_{1500}$/P-GUS-NOS 3' expression, the 1500 bp TA29 promoter fragment is capable of directing anther specific expression of GUS

in individual transformed tobacco plants. Two of the plants with anther specific GUS expression showed much higher lev ls of GUS activity in the anthers than seen for any of the $TA29_{500}$/P-GUS-NOS 3' transformants.

The anther specific GUS expression was further characterized as being limited to tapetal cells by incubating anthers in 0.1 mM $NaPO_4$ buffer, pH 7.0, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 10 mM Na_2EDTA containing 1 mg/mL X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide). This GUS substrate releases a blue product upon GUS hydrolysis. Microscopic observation showed that the blue staining was limited to the tapetal cells in the anther. Wild-type control tobacco anthers showed no blue staining.

To verify that anthers of buds at stages 3 and 4 show the peak $TA29_{500}$/P-GUS-NOS 3' expression levels, a time course of GUS activity was established in anthers from two plants exhibiting anther specific GUS expression. Anthers from buds of stages 2,3,4, and 5 were assayed for GUS enzyme activity using the fluorescence assay described above. In each case the activity was highest at stage 3.

Seeds were collected following self-fertilization of individual transformants. To determine the number of genetic loci for the $TA29_{1500}$/P-GUS-NOS 3' gene, and linked kanamycin resistance marker, seeds were germinated as described above. A single locus for the trait was present in five out of nine transformants with anther specific GUS expression that were tested. The remaining transformants exhibited a ratio which was greater than 3:1, indicating the presence of more than one integration site.

To establish lines of plants homozygous for the $TA29_{500}$/P-GUS-NOS 3' gene 4-7 kanamycin resistant plants from lines showing 3:1 segregation in the seed

germination test were potted in soil and grown to maturity in the greenhouse. Seed was collected after self fertilization, and seeds from the progeny of two lines were germinated on kanamycin containing medium. A population of seed that is 100% resistant was identified for each line thereby establishing these as homozygous populations.

EXAMPLE 5

The chimeric $TA29_{1500}$/P-GUS-NOS 3' gene was introduced into tomato (Lycopersicum esculentum, var. Herbs red cherry) by Agrobacterium tumefaciens infection of cotyledon explants with the Agrobacterium harboring pZ6ALG that was described above. Primary transformants were analyzed to demonstrate anther specific expression of this gene.

Standard aseptic techniques for the manipulation of sterile media and axenic plant/bacterial cultures were followed, including the use of a laminar flow hood for all transfers. Ten days prior to inoculation, tomato seeds were surface sterilized in a solution of 10% Clorox® and 0.1% SDS, then rinsed 3 times with sterile water. Seeds were planted in magenta boxes containing OMS agar medium (Table 2) and germinated in a culture room under mixed fluorescent and grow lights with a 16 hour day length at 25°C.

A single colony of Agrobacterium harboring pZ6ALG was used to inoculate 3 mL of MinA (Table 1) broth and it was grown at 25°C with shaking over two nights. The culture was diluted to an OD_{650} of 0.1 in a flask containing 30 mL of MinA broth and grown with shaking at 28°C to a density equal to an OD_{650} of 0.3. Using a sterile scalpel, cotyledons were excised from seedlings and approximately 2 mm of tissue was removed from each end. These explants were planted underside down at 10 per plate on CTM agar (Table 2) containing 75 µM

acetosyringone in dimethylsulfoxide (DMSO). Then, 5 mL of the Agrobacterium solution was added to each plate, making sure to wet all of the explants, and then removed. The plates were incubated in the culture room for 2-3 days. Thereafter, 15 mL of OMS solution (Table 2) containing 500 mg/L cefotaxime was added to each plate and incubated for 10-15 minutes. The explants were then transferred to sterile filter paper to remove excess liqiud, then to plates of CTM medium containing 500 mg/L cefotaxime and 50 mg/L kanamycin, and incubated in the culture room for approximately 3 weeks. Explants were then transferred to fresh plates of CTM medium with 0.1 mg/L zeatin, 500 mg/L cefotaxime and 50 mg/L kanamycin and incubated in the growth room for 2-3 weeks. Fully formed shoots were excised, dipped in 1 mg/L indole butyric acid, and planted in magenta boxes containing 85 mL OMS gelrite (Table 2) with 500 mg/l cefotaxime for root formation.

To test the shoots for stable transformation, the ability of leaf tissue to reshoot on kanamycin was assayed. A leaf was removed from each shoot, cut into 2-3 pieces, and partially embedded in TB agar medium (Table 2) with and without 25 mg/L kanamycin. Three to four weeks after the shoots were excised, the ones that showed kanamycin resistance in both the rooting and leaf shooting assays were planted in 8 inch pots in Metro-Mix, covered with a plastic bag (for a week), and grown in a growth chamber set at 24°C, 12 hour daylength. After several weeks, the plants were transferred to the greenhouse and grown to maturity. Expression of the $TA29_{1500}$/P-GUS-NOS 3' gene was assayed using the X-Gluc GUS enzyme assay described above. Immature buds were excised, dissected into pieces, and incubated in the X-Gluc solution overnight. Blue staining was observed only in the anthers of buds from all 6 of the plants

tested. S eds were collected following self-fertilization of each transformant. Single locus homozygous lines were identified as described in Example 3.

TABLE 2

OMS Medium	
Per liter:	1 pkg MS Minimal Organic Medium without sucrose (Gibco)
	3 g sucrose
	1 ml of B5 Vitamins
	3 mM MES, [2-(N-morpholino)ethane-sulfonic acid]
	pH 5.7
	8 g agar (for gelrite: add 0.75 g MgCl
and	2 g gelrite instead of agar)
CTM Medium	
Per liter:	1 pkg MS Minimal Organic Medium without sucrose (Gibco)
	3 g sucrose
	1 ml B5 vitamins: 1mg/mL nicotinic acid, 10 mg/mL thiamine hydrochloride, 1mg/mL pyridoxine hydrochloride, 100 mg/mL M-inositol
	3 mM MES
	pH 5.7
	7 g agar
	autoclave and add 1 mL of 1 mg/mL zeatin
TB Medium	
Per liter:	1 pkg MS medium without sucrose
	1 g sucrose
	1 ml of B5 Vitamins
	1 ml of 1 mg/mL BAP

0.4 mL of 1 mg/mL indole acetic acid (IAA)
pH 5.8
8 g agar

EXAMPLE 6

The chimeric $TA29_{1500}$/P-GUS-NOS 3' gene was introduced into Brassica napus (cv. Westar) by Agrobacterium tumefaciens infection of hypocotyl explants with the Agrobacterium harboring pZ6ALG that was described above. Primary transformants were analyzed to demonstrate anther specific expression of this gene.

Standard aseptic techniques for the manipulation of sterile media and axenic plant/bacterial cultures were followed, including the use of a laminar flow hood for all transfers. Brassica seeds were surface sterilized in a solution of 10% Clorox® and 0.1% SDS, then rinsed thoroughly with sterile water. Seeds were planted in 190 mm crystallization dishes containing 30 mM calcium chloride, 1.5% agar, and placed in the dark at 25°C for five days.

A single colony of Agrobacterium harboring pZ6ALG was used to inoculate 3 mL of MinA (Table 1) broth and it was grown at 28°C with shaking for 18-20 hours. The culture was grown to an OD_{650} of 1.0-2.0. Then 22.5 mL of bacterial dilution medium (1 package MS minimal organic medium and 30 g sucrose per liter) containing 100 µM acetosyringone was placed in a sterile dish. Using a sterile scalpel, the seedling hypocotyls were cut into 1 cm segments and placed immediately into the bacterial dilution medium. 2.5 mL of the Agrobacterium culture was added and after a 30 minute incubation the hypocotyl pieces were placed on plates containing co-cultivation medium: BC-1 (Table 3) containing 100 µM

acetosyringone. These plates were incubated in dim light for 3 days at 25°C. The hypocotyl pieces were then transf rr d to plat s of BC-1 medium containing 200 mg/L carbenicillin and 50 mg/L kanamycin and incubated in the culture room. After 3, 6, and 9 weeks, calli growing from the cut hypocotyl ends were transferred to plates of BS-48 shoot regeneration medium (Table 3) containing 200 mg/L carbenicillin, and incubated in continuous light at 25°C. The explants were transferred to fresh medium of the same composition every 2 weeks. Shoot primordia appeared 4-6 weeks after transfer to BS-48 medium and after elongating they were transferred to MSV-1A medium (Table 3) and incubated with a 10-12 hour photoperiod. After 3 weeks each shoot tip along with several internodes was transferred to fresh MSV-1A medium and incubated for another 3 weeks. The shoots were then cut near the agar surface, dusted with Rootone to induce root formation, planted in 8 inch pots in Metro-Mix, covered with a plastic bag (for 2 weeks), and grown in a growth chamber set at 24°C, 16 hour daylength. After several weeks, the plants were transferred to the greenhouse and grown to maturity.

Expression of the $TA29_{1500}$/P-GUS-NOS 3' gene was assayed using the X-Gluc GUS enzyme assay described above. Immature buds were excised, cut into pieces, and incubated in the X-Gluc solution overnight. Blue staining was observed in the anthers and not in other parts of buds from all 4 of the plants tested. Seeds were collected following self-fertilization of each transformant.

TABLE 3

BC-1	
Per liter:	1 pkg MS Minimal Organic Medium (Gibco)
	30 g Sucrose
	18 g Mannitol
	0.2 mg 2,4-D
	3 mg Kinetin
	6 g DNA-Grade Agarose
	pH 5.8
BS-48	
Per liter:	MS Minimal Organic Medium
	1 mL B5 Vitamins: 1 mg/mL nicotinic acid, 10 mg/mL thiamine hydrochloride, 1mg/mL pyridoxine hydrochloride, 100 mg/mL M-inositol
	250 mg Xylose
	10 g Glucose
	0.6 g MES
	4 g DNA Grade Agarose
	pH 5.7
	2 mg Zeatin (Add after autoclaving)
	0.1 mg IAA (Add after autoclaving)
MSV-1A	
Per liter:	MS Minimal Organic Medium
	10 g Sucrose
	1 mL B5 Vitamins
	6 g DNA-Grade Agarose
	pH 5.8

EXAMPLE 7

A. Isolation and Characterization of Anther-Specific cDNA from Brassica

To obtain promoters from Brassica that are capable of directing GUS expression specifically in anthers, anther-specific cDNAs were isolated and characterized.

A cDNA library of Brassica napus (cv. Westar) was constructed using poly(A)$^+$-RNA isolated from developing anthers dissected from 2-3 mm flower buds. Total and poly(A)$^+$-RNAs were purified using RNA Extraction and mRNA Purification Kits (Pharmacia) according to the manufacturer's specifications. cDNA was synthesized using the RiboClone™ cDNA Synthesis System (Promega), ligated with EcoR1 adaptors (Promega), inserted into the EcoR1 site of the Lambda ZAP II vector (Stratagene), and packaged into Lambda particles using the GIGAPACK™GOLD Lambda DNA Packaging Extracts (Strategene). This library was used for differential screening using the standard phage plaque-lift method (Bio-Rad). The probes were ^{32}P-labeled cDNA generated both from (1) poly(A)$^+$-RNA purified from developing anthers and (2) poly(A)$^+$-RNA purified from seedlings. The putative positive Lambda ZAP II clones were prepared to rescue the encompassed pBluescript plasmid according to the supplier's instructions (Stratagene). Six anther specific cDNA clones [p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7)] in the pBluescript vector (Stratagene) were identified by Southern and Northern blots (Maniatis et al. 1982).

To verify the anther-specificity of the isolated cDNAs and further characterize their expression, RNA blots [Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982)] containing total RNA purified from seedlings, petals, filaments, different stages of anthers and pistils were hybridized with the ^{32}P-labeled cDNA insert prepared from each clone. The mRNAs encoded by p112 and p158 accumulated very early in the development of anthers, in flower buds less than 2 mm in length, and the levels of message declined and disappeared during anther

maturation. In contrast, the levels of mRNA encoded by p54 and p73 cDNAs increased during anther development and reached the peak level at anthesis. No hybridization signal was observed in seedling, petal, filament, and pistil RNAs. The expression of p42 and p42W also showed anther specificity by Northern blot analysis.

The techniques of *in situ* hybridization were used to determine the tissue localization of mRNAs represented by the cloned cDNAs in anthers of *Brassica*. The techniques used are described by Cox and Goldberg (1988, in *Plant Molecular Biology: A Practical Approach*, C. H. Shaw, ed. Oxford: IRL Press, pp. 1-34).

Tissue sections were prepared from *Brassica napus* anthers dissected from different stages of flower buds. Sense and antisense probes were prepared from the cDNA clones using the polymerase chain reaction (GeneAmp kit, Perkin-Elmer Cetus) with universal and reverse primers (Pharmacia), followed by preparation of ^{35}S-labeled transcripts synthesized by T3 and T7 RNA polymerase using methods suggested by the supplier of the pBluescript vector (Stratagene). The p112 antisense probe hybridized only to the tapetal cell layer surrounding the developing pollen grains and not to any other cells in the section indicating that the p112 mRNA is tapetal-cell specific. The control p112 sense probe did not show any specific hybridization. Hybridization of the p158 antisense probe also showed the p158 mRNA to be tapetum-specific, but the signal level was lower than that of p112. These results were consistent with the results of the Northern blots in which the messages of p112 and p158 accumulated at early stages of anther development and disappeared when the tapetum layer was degenerated. Hybridization of the p54 antisense probe showed pollen-specific

expression of p54 mRNA, which accumulated to a maximum level in mature poll n. The in situ hybridization result for p42 show d that the mRNA was localized in the outer parenchymatic cells of the vascular bundle, the tapetal cell layer and pollen grains, but not in the connective tissue or the outer cells of the exotecium. The in situ localization of p73 mRNA was not examined, but its message accumulated at the maximum level in the mature anther indicating it could be another pollen-specific gene.

B. Determination of DNA sequence of anther-specific cDNA clones

DNA from cDNA clones p42, p42W, p54, p73, p112 and p158 was made for sequence analysis by purifying double-stranded plasmid using standard procedures [Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982)]. The nucleotide sequences of the cDNAs were determined by the dideoxy method using a ^{T7}Sequencing™ kit (Pharmacia). Adjacent regions of the clones were sequenced by priming with synthetic oligonucleotides designed from sequences obtained from previous gel readings.

The cDNA and deduced amino acid sequences of p42 showed significant homology with chalcone synthase from mustard [Ehmann and Schaefer, Plant Mol. Biol. 11:869-870 (1988)], parsley [Reimold et al. EMBO J. 2: 1801-1805 (1983)] and soybean [Akada et al. Nucleic Acids Res. 18:3398 and 5899 (1990)]. The cDNA sequences of p42W and p112 share 98% identity, and p42W has 18 extra nucleotides at the 5'-terminus and 399 extra nucleotides at the 3'-terminus. The sequences of the cDNA clones are listed as SEQ ID NOS:2-7 for p73, p112, p54, p42W, p42 and p158, respectively, and the

characterization for the Brassica napus anther-specific cDNA clones is summarized in Tabl 4.

C. Isolation of Anther-Specific Genomic Clones and Promoters

A Brassica napus genomic library of genes corresponding to the isolated and characterized cDNA clones are screened, by anther-specific cDNA inserts which are ^{32}P-labeled as probes, using the standard phage plaque-lift method (Bio-Rad). After the isolation of a genomic clone coding for the anther-specific cDNA, primer extension experiments [Ausubel et al.], Current Protocols in Molecular Biology, John Wiley & Sons, New York (1987)] are performed to determine the transcription start site. The anther-specific promoter is then isolated as the DNA segment located 5' to the transcription start site. In this way, novel Brassica napus tapetum and pollen specific promoters are prepared and used to construct chimeric genes for anther-specific expression of GUS. The promoter fragment is placed adjacent to the GUS-NOS 3'construction in pTZALG, after removing the TA29 promoter fragment. The resulting chimeric genes: 42/P-GUS-NOS 3', 42W/P-GUS-NOS 3', 54/P-GUS-NOS 3', 73/P-GUS-NOS 3', 112/P-GUS-NOS 3', and/or 158/P-GUS-NOS3' are each cloned into pZS96. The genes are then introduced into tobacco, tomato, and/or Brassica as described previously, and GUS expression is analyzed in transformants as described in Example 9.

TABLE 4

Brassica napus Anther-Specific cDNAs

Clone	cDNA (kb)	mRNA[a] (kb)	Gene[b] copy #	In situ Localization	Homology
1. p42	0.75	~0.8	2-3	Tapetum, Pollen Periphery of VB[d]	Chalcone Synthase
2. p42W	0.97	~0.8	2-3	--	--
3. p54	0.41	~1.5	4-8	Pollen	--
4. p73	0.34	~0.8	3-5	--	--
5. p112	0.55	~0.8	2-3	Tapetum	--
6. p158	0.91[c]	~0.8	1-2	Tapetum	--

[a]Size estimated on Northern blots

[b]Copy number determined on Southern blots of genomic DNA

[c]p158 cDNA insert size estimated on agarose gel

[d]VB = vascular bundle

EXAMPLE 8

Chimeric genes for expression of GUS specifically in pollen can be constructed by making use of other pollen specific promoters known to one skilled in the art. LAT52 is a gene from tomato that is expressed preferentially in the pollen. The identification, cloning, and characterization of this gene is described by Twell et al., Mol. Gen. Genet. 217: 240-245 (1989). Genes that are expressed specifically in pollen have also been studied in Zea mays and Tradescantia paludosa by Stinson et al., Plant Physiol. 83: 442-447 (1987). In Hanson et al., The Plant Cell 1: 173-179 (1989) the isolation of the pollen specific Zmc13 gene is mentioned. To express GUS in pollen, a fragment containing the LAT52 or Zmc13 promoter region is prepared from the clone of the gene and placed adjacent to the GUS-NOS 3' construction in pTZALG, after removing the TA29 promoter fragment. The resulting

chimeric genes LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-NOS 3' are each cloned into pZS96 creating pZ6TGN and pZ6CGN, respectively.

EXAMPLE 9

The chimeric LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-NOS 3' genes, described above, are introduced into tobacco by infection of leaf disks with Agrobacterium tumefaciens harboring pZ6TGN and pZ6CGN, respectively. The procedure is carried out as described in Example 3. Kanamycin-resistant transformants are tested for pollen specific expression of GUS by the enzyme assays described in Examples 4 and 5. Pollen is collected from mature anthers, incubated in X-Gluc solution, and blue staining of the pollen is assessed microscopically. Alternatively, a protein extract is prepared from collected pollen and assayed using the MUG substrate. Samples of ovule and leaf tissue are also tested for GUS activity as described above to determine the specificity of expression in the pollen. Seed is collected from plants showing anther specific GUS activity, and homozygous lines are established as described in Example 3.

The chimeric LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-NOS 3' genes are introduced into tomato by infection of cotyledon explants with Agrobacterium tumefaciens harboring pZ6TGN and pZ6CGN, respectively. The procedure is carried out as described in Example 5. Kanamycin-resistant transformants are tested for pollen specific expression of GUS by the enzyme assays described in Examples 4 and 5. Pollen is collected from mature anthers, incubated in X-Gluc solution, and blue staining of the pollen is assessed microscopically. Alternatively, a protein extract is prepared from collected pollen and assayed using the MUG substrate. Samples of ovule and leaf tissue are

also tested for GUS activity as described above to determine th specificity of expression in th pollen. Seed is collected from plants showing anther-specific GUS activity, and homozygous lines are established as described in Example 3.

The chimeric LAT52/P-GUS-NOS 3' and Zmc13/P-GUS-NOS 3' genes are introduced into Brassica by infection of hypocotyl explants with Agrobacterium tumefaciens harboring pZ6TGN and pZ6CGN, respectively. The procedure is carried out as described in Example 6. Kanamycin-resistant transformants are tested for pollen-specific expression of GUS by the enzyme assays described in Examples 4 and 5. Pollen is collected from mature anthers, incubated in X-Gluc solution, and blue staining of the pollen is assessed microscopically. Alternatively, a protein extract is prepared from collected pollen and assayed using the MUG substrate. Samples of ovule and leaf tissue are also tested for GUS activity as described above to determine the specificity of expression in the pollen. Seed is collected from plants showing anther-specific GUS activity, and homozygous lines are established.

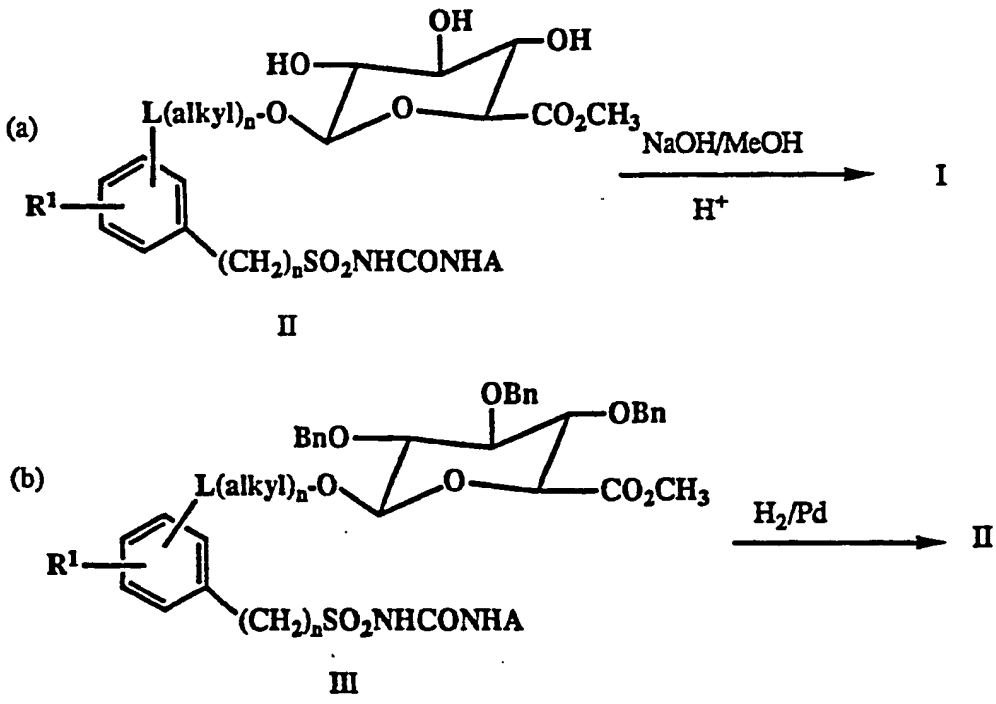
Preparation of the Protoxin

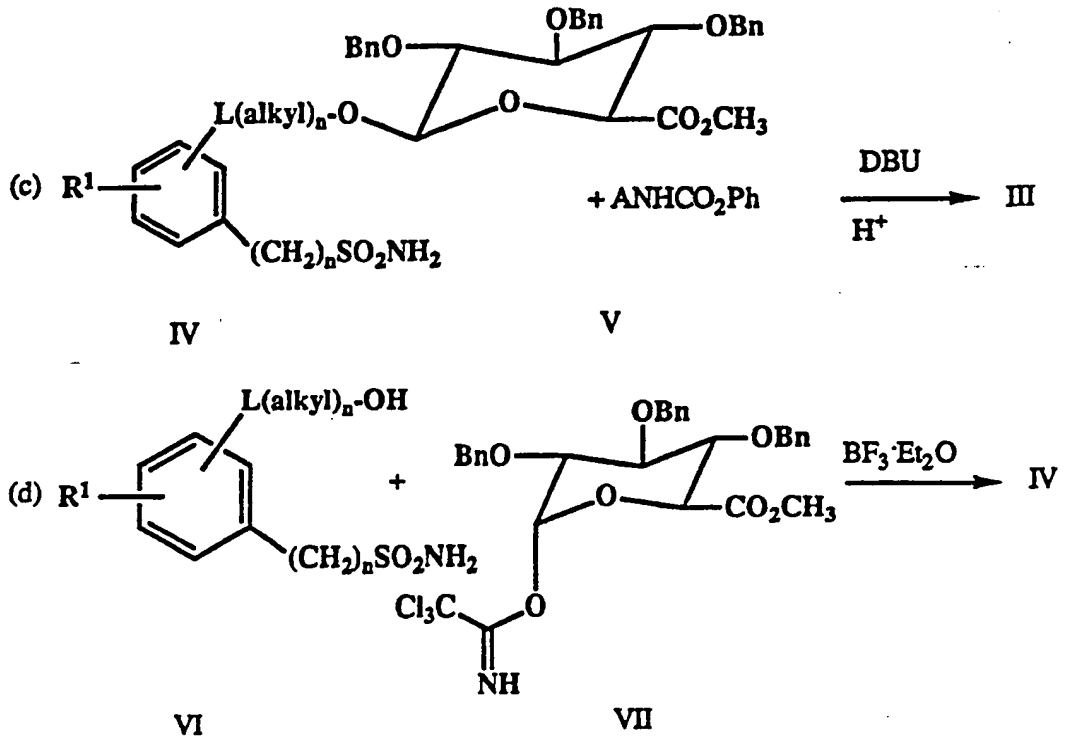
To form a β-O-glucuronide, the only structural requirement on the aglycone is a hydroxyl or oxyanion residue. As long as the residue is at least weakly nucleophilic and is not severely sterically hindered, conjugation can be performed by one or more of the means outlined below. Examples of suitable substrates include, but are not limited to, phenols, salts of phenols, organic alcohols, salts of organic alcohols, suitably activated carbonyl compounds such as 1,3-dicarbonyls, imides, and activated secondary amides, and so forth. A variety of compounds that are

phytotoxic contain hydroxyl groups. Some of thes compounds have been made into glucuronides and tested on transgenic plants expressing male organ-specific GUS.

The compounds of Formula I can be prepared by either of two methods, differing by choice of glucuronidating reagent. The first, Method A, utilizes the reagent of Formula VII, whose synthesis is described by Schmidt et al. in Synthesis, p. 885 (1981). Method A is outlined in Scheme 1 for compounds of Formula I wherein J is J-1 but this method is equally applicable to compounds wherein J is J-2 to J-5.

Scheme 1





wherein:

n, R, R^1, and A are as previously described, and Bn = CH_2Ph.

The reaction of Scheme 1(a) can be carried out by contacting a glucuronide methyl ester of Formula II with 2-5 equivalents of sodium hydroxide in methanol at a temperature between 0 and 40°C for 0.1 to 24 hours. The product can be isolated by adding a sufficient amount of a cationic ion-exchange resin to protonate the glucuronic acid sodium salt, filtering the resin, and evaporating the solvent.

The reaction of Scheme 1(b) can be carried out by the hydrogenolysis of a methanolic solution of a 2,3,4-tri-O-benzyl glucuronide methyl ester of Formula III with a palladium catalyst, such as 20% palladium hydroxide on carbon (Pearlman's catalyst) at 0 to 30°C

for 0.1 to 24 hours under a hydrogen pressure of 1-10 atmospheres. The product can be isolated by filtration and removal of solvent.

The reaction of Scheme 1(c) can be carried out by contacting a protected sulfonamide glucuronide of Formula IV with an N-heterocyclyl carbamic acid phenyl ester of Formula V, examples of which are well-known in the art and which are known to form highly herbicidal sulfonylureas with suitable sulfonamides. Generally, equimolar quantitites of compounds of Formula IV and V are dissolved in an inert solvent such as acetonitrile, 1 to 2 equivalents of an amidine base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) are added at -10 to 40°C, and the mixture is allowed to react for 0.1 to 2 hours, after which time the product is isolated by acidification with aqueous acid and filtration or extraction into a suitable organic solvent. Purification can be accomplished by recrystallization or chromatography on silica gel.

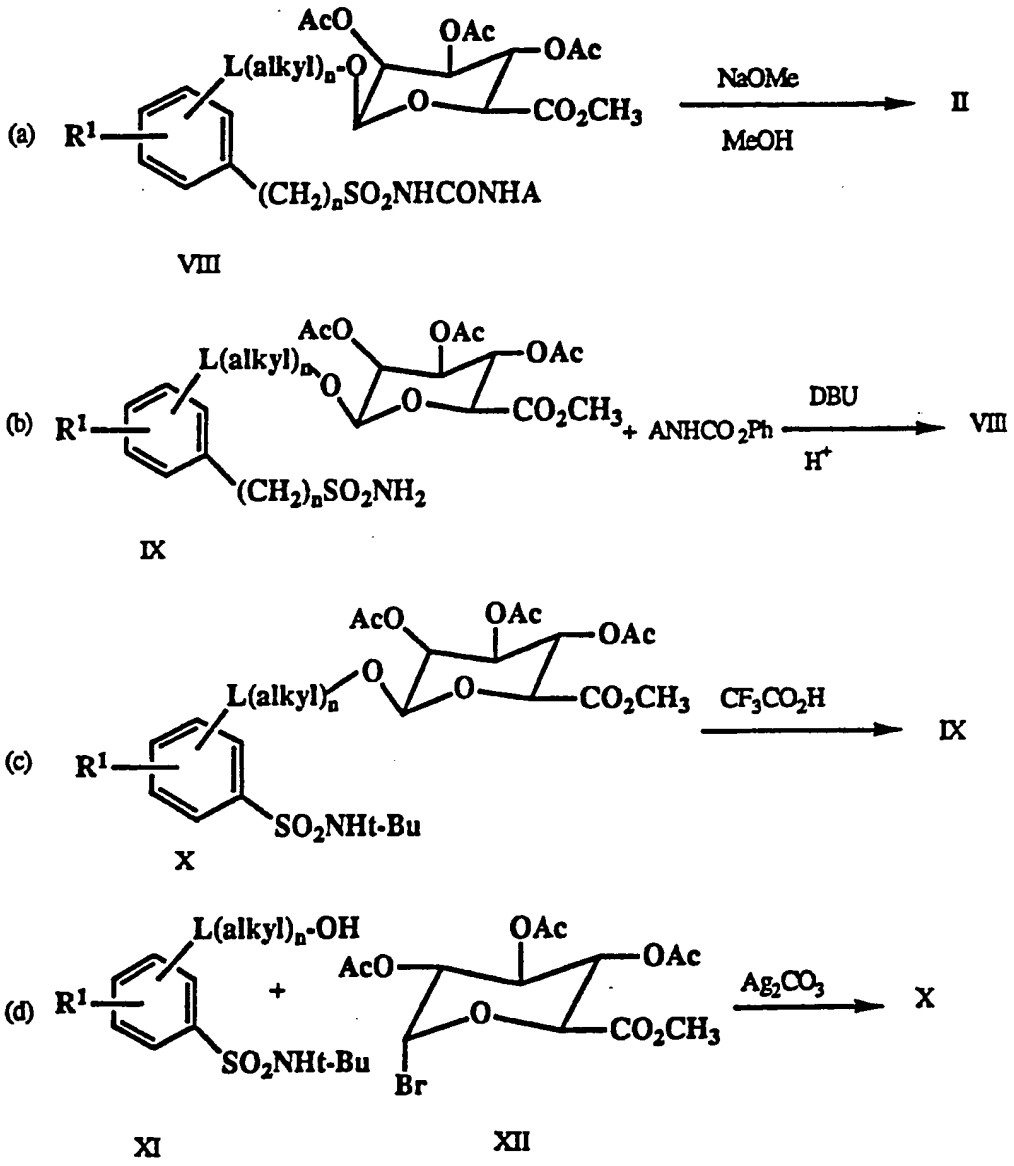
In the reaction of Scheme 1(d), the compound of Formula IV can be prepared by the selective O-glycosylation of a hydroxy-substituted sulfonamide with the aforementioned reagent VII in the presence of an acidic catalyst, such as boron trifluoride etherate in a suitable solvent, such as dichloromethane, at -40 to 0°C for 0.1 to 2 hours. The product can be isolated by washing the organic solution with aqueous $NaHCO_3$, removing the solvent, and by chromatography on silica gel and/or recrystallization.

The preparation of hydroxylated sulfonamides can be accomplished by a wide variety of methods well-known to one skilled in the art.

An alternative procedure, Method B, utilizes a commercially-available glucosylating reagent, acetobromo-α-D-glucuronic acid methyl ester (XII), and

is outlined in Scheme 2 for the preparation of compounds of Formula I wherein J is J-1. This method works qually well for compounds of F rmula I wherein J is J-2 to J-5.

Scheme 2



wherein:

n, R, R^1, are as previously defined and Ac is acetyl.

The reaction of Scheme 2(a) can be carried out by contacting a solution of a compound of Formula VIII in methanol with a catalytic amount of sodium methoxide (0.01 to 0.1 equivalents) for 0.1 to 10 hours at 0 to 30°C. The product can be isolated by neutralization of the catalyst and concentration of the reaction mixture and may optionally be purified by chromatography.

The reaction of Scheme 2(b) can be carried out exactly as described above for the reaction of Scheme 1(c). The reaction of Scheme 2(c) can be carried out by contacting a compound of Formula X with trifluoroacetic acid which can be used as a solvent at 0-40°C for 0.1 to 10 hours. The product of Formula IX can be isolated by removal of excess trifluoroacetic acid under vacuum and chromatography on silica gel.

The reaction of Scheme 2(d), can be accomplished by contacting a hydroxylated t-butyl sulfonamide of Formula XI with 1-2 equivalents of methyl 2,3,4-triacetyl-1-bromo-α-D-glucuronate and 1-3 equivalents of a silver salt such as silver carbonate in an inert anhydrous solvent such as benzene or dichloromethane for 1-100 hours. The product can be isolated by filtration followed by chromatography of the filtrate on silica gel.

The preparation of t-butyl sulfonamides containing hydroxylated side-chains can be accomplished by a variety of methods well-known to one skilled in the art.

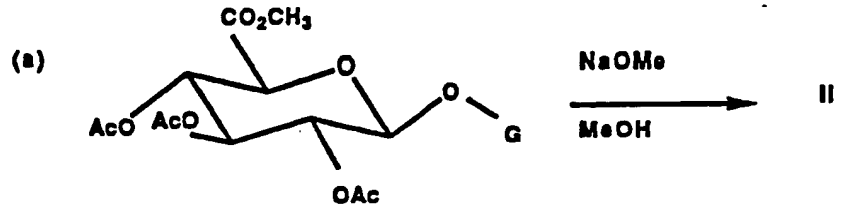
A third glucosylating method, Method C, can be employed utilizing the commercially-available reagent

acetobromo-α-O-glucuronic acid, methyl ester (XII) and is outlined in Scheme 3.

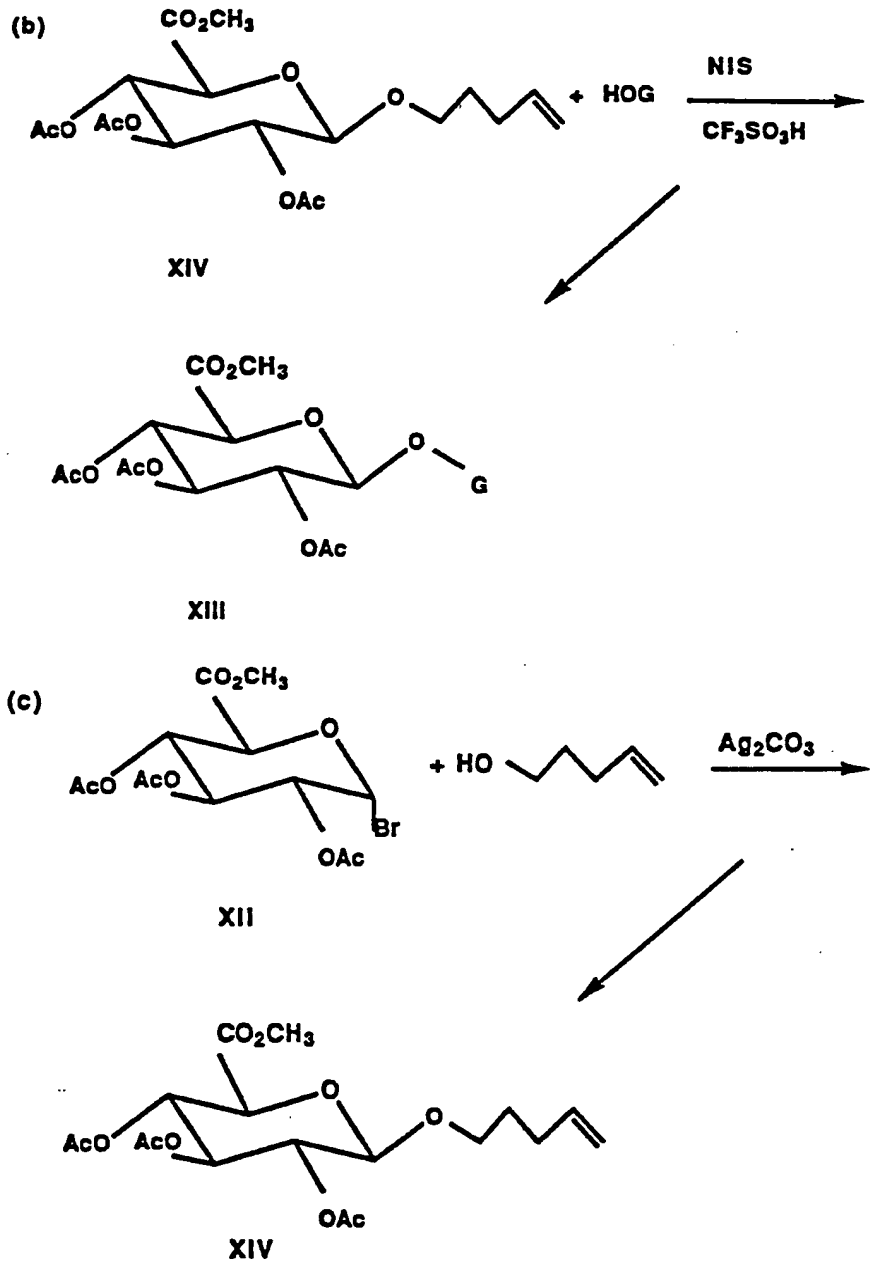
The reaction of Schem 3(a) can be carried out as described above for the reaction of Scheme 2(a). The reaction of Scheme 3(b) can be carried out by contacting XIV with a chemcial containing a hydroxyl group, in the presence of 1-2 equivalents of N-iodosuccinimide (NIS) and 0.5-1.5 equivalents of a strong acid such as trifluoromethanesulfonic acid under an inert atmosphere such as nitrogen, and in an inert anhydrous solvent such as dichloromethane for 0.1-10 h (Konradsson, et al; Tetrahedron Letter, Vol. 31 (30), 4313 (1990)). The product could be isolated by washing the organic solution with aqueous $NaHCO_3$ and aqueous $Na_2S_2O_3$ solution, removing the solvent, and by chromatography on silica gel.

The reaction of Scheme 3(c) can be carried out by contacting XII with 1-10 equivalents of 4-penten-1-ol in the presence of 1-2 equivalents of an inorganic metal salt such as silver carbonate, at 0 to 40°C in an anhydrous inert solvent such as benzene for 1-40 h. The product is obtained by filtering the solid, removing the solvent and by recrystallization or chromatography on silica gel.

Scheme 3



XIII

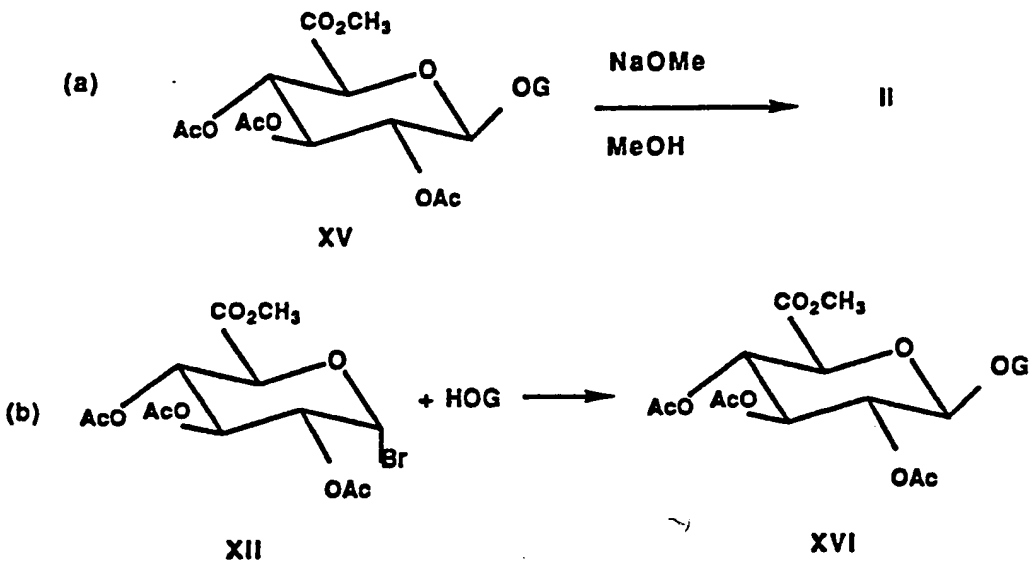


wherein G represents an aglycone toxin.

Another method to prepare the protoxins of the instant invention is described in Scheme 4.

The reaction of Scheme 4(a) can be carried out as describ d above for the reaction of Scheme 2(a). The reaction of Scheme 4(b) can be carried out by contacting XII with a chemical containing a hydroxyl group in the presence of 1-5 equivalents of an inorganic metal salt such as mercuric cyanide at 0-140°C, in an inert anhydrous solvent such as dimethylformamide for 0.1-10 h. The product is obtained by partitioning the reaction mixture between water and an organic solvent such as ethyl acetate, combining the organic extracts and removing the solvent. Purification is by silica gel chromatography.

Scheme 4



wherein G or HOG represent an aglycone toxin.

The following Examples illustrate the general methods outlined above.

EXAMPLE 10

[3-[2-[[[[(4,6-Dimethoxy-2-pyrimidinyl)-amino]carbonyl]amino]sulfonyl]phenoxy]-propyl]-β-L-gluco-pyranosiduronic acid

A. A solution of 0.65 g of 2-(3-hydroxypropoxy)-benzenesulfonamide and 2.2 g of compound VII in 70 mL of dichloromethane was cooled to -25°C under a nitrogen atmosphere and 5 drops of boron trifluoride etherate was added, and the mixture was stirred for 1/2 hour at -20°C. Aqueous $NaHCO_3$ was added, the CH_2Cl_2 layer was separated, washed with brine, dried with $MgSO_4$, concentrated, and chromatographed on silica gel eluting with an ethyl acetate-hexane mixture to provide 0.30 g of the non-crystalline glucoside which was not further purified.

B. To a solution of 0.30 g of the glucoside from step A in 5 mL of acetonitrile was added 0.10 g of phenyl N-(4,6-dimethoxy-2-pyrimidinyl)carbamate, followed by 0.10 g of DBU. The mixture was stirred at ambient temperature for 10 minutes, and was then made acidic with aqueous oxalic acid. The product was extracted with ethyl acetate, washed with brine, dried with $MgSO_4$ and concentrated. Application of the crude product to a column of silica gel and elution with a mixture of ethyl acetate and hexanes, and finally with pure ethyl acetate afforded 0.3 g of the nearly pure sulfonylurea. NMR ($CDCl_3$) δ: 2.0 (multiplet, 2H), 3.4-4.2 (multiplet), 3.7 (singlet, 3H), 3.9 (singlet, 6H), 5.8 (singlet, 1H), 6.8 (doublet, 1H), 7.1 (triplet, 1H), 7.3 (multiplet), 7.5 (triplet, 1H), 8.2 (doublet of doublet, 1H). This was without additional purification in step C.

C. To a solution of 0.30 g of the product of step B in 15 mL of methanol was added 0.15 g of 20% palladium hydroxide-on-carbon (Pearlman's catalyst) and the

mixture was stirred under a hydrogen atmosphere for 2 hours at ambient temperature. The catalyst was removed by filtration and the solution f the product was concentrated and partially purified by chromatography on silica gel, eluting with a gradient of methanol in dichloromethane.

The intermediate methyl ester was saponified by dissolving it in 10 ml of methanol and adding 1 mL of a solution of 1N NaOH in 9 ml MeOH. After allowing the solution to stand for 2 1/2 hours at ambient temperature, it was stirred with excess ion-exchange resin (acid-form of polystyrene-sulfonic acid resin), filtered, and concentrated to provide 0.04 g of the title compound. Fast-atom bombardment (FAB) mass spectrum: 589 ($M^+ + 1$).

EXAMPLE 11

[2-[2-[[[[(4-Chloro-6-methoxy-pyrimidin-2-yl)-amino]carbonyl]amino]sulfonyl]phenyl]ethyl]-β-D-glucopyranosiduronic acid

A. To methyl 2,3,4-triacetyl-1-bromo-α-D-glucuronate (6 g, 15.11 mmole) in anhydrous benzene (400 mL) at room temperature under N_2 atmosphere, N-(1,1-dimethylethyl)-2-(2-hydroxyethyl)benzenesulfonamide (4 g, 10.11 mmole) was added, followed by silver carbonate (5 g, 18 mmole). The mixture was stirred at room temperature for 24 hours. Another portion of methyl 2,3,4-triacetyl-1-bromo-α-D-glucuronate (6 g, 15.11 mmole) and silver carbonate (5 g, 18 mmole) were added. The mixture was further stirred for 24 hours and was then suction filtered. The solid was washed with benzene (100 mL). The filtrate and washings were combined and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (EtOAc/hexane = 1:1) to give the product as an oil (5.5 g, 63% yield).

NMR ($CDCl_3$) δ: 1.23 (singlet, 9H), 1.95 (singlet, 3H), 2.04 (singlet, 6H), 3.10-3.63 (multiplet, 2H), 3.86 (singlet, 3H), 3.90-4.25 (multiplet, 3H), 4.68 (doublet, J = 7 Hz, 1H), 4.95-5.33 (multiplet, 3H), 7.28-7.57 (multiplet, 3H), 8.00-8.20 (multiplet, 1H).

B. The product from step A (200 mg, 0.35 mmole) in trifluoroacetic acid (5 mL) was stirred at room temperature for 45 minutes. Trifluoroacetic acid was removed under reduced pressure at room temperature. The syrupy residue was redissolved in n-butylchloride (10 ml) and concentrated under reduced pressure. This was repeated once more to give a clear syrup which was purified by silica gel column (eluted with ethyl acetate) chromatography to give 0.160 gm (88%) of the desired product as a colorless oil.

NMR ($CDCl_3$) δ: 1.70 (singlet, 3H), 1.98 (singlet, 3H), 2.00 (singlet, 3H), 3.35 (doublet of triplet, J_1 = 5 Hz, J_2 = 15 Hz, 1H), 3.74 (singlet, 3H), 3.83 (multiplet, 1H), 4.05 (doublet, J = 10 Hz, 1H), 4.30 (multiplet, 1H), 4.48 (doublet, J = 8.5 Hz, 1H), 4.85 (triplet, J = 9.5 Hz, 1H), 5.00-5.15 (multiplet, 4H), 7.29-7.53 (multiplet, 3H), 8.05 (doublet, J = 7.5 Hz, 1H).

C. To the product from step B (6.5 g, 12.29 mmole) in acetonitrile (300 mL), the phenylcarbamate (3.44 g, 12.30 mmole) was added. The solution was cooled with an ice bath and diazabicyclo[5.4.0]-undec-7-ene (2 mL) was added. The reaction solution was stirred for 2 hours and was concentrated under reduced pressure. The residue thus obtained was purified by silica gel column chromatography (solvent system: $MeOH/CH_2Cl_2$ = 1:4) to give the product as an oil (6.5 g, 74%).

NMR (DMSO) δ: 1.90 (singlet, 3H), 1.94 (singlet, 3H), 1.96 (singlet, 3H), 3.05-4.03 (multiplet, 4H), 3.62 (singlet, 3H), 3.70 (singlet, 3H), 4.02 (doublet, J = 6 Hz, 1H), 4.85-5.00 (multiplet, 3H), 5.17 (triplet, J =

7 Hz, 1H), 6.05 (singlet, 1H), 6.75 (doublet, J = 6 Hz, 1H), 7.00-7.20 (multiplet and a broad singlet, 2H), 7.30-7.50 (multiplet and a broad singlet, 2H), 7.83 (doublet, J = 5 Hz, 1H).

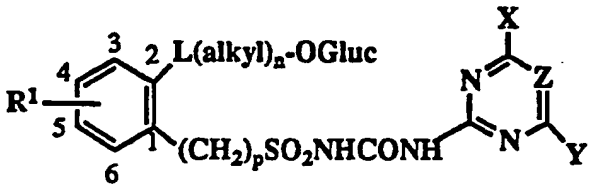
D. The product from step C (0.1 g, 0.14 mmole) was dissolved in methanol (10 ml). A catalytic amount of NaOMe in MeOH was added. The reaction solution was stirred at room temperature for 1 hour. A drop of acetic acid was added. The solvent was removed under reduced pressure at room temperature. The residue thus obtained was purified on a silica gel column (eluted with 10% MeOH in CH_2Cl_2) to give the desired product (a foam, 74 mg, 90%).
NMR (DMSO) δ: 2.90-3.95 (multiplet, 7H), 3.59 (singlet, 3H), 3.78 (singlet, 3H), 4.08 (multiplet, 2H), 4.30 (doublet, 5 Hz, 1H), 5.03-5.10 (broad singlet, 1H), 5.12-5.20 (broad singlet, 1h), 5.23 (doublet, J = 4 Hz, 1h), 6.40 (singlet, 1H), 7.12-7.30 (multiplet, 3H), 7.91 (doublet, J = 5 Hz, 1H), 8.70 (broad singlet, 1H).

E. The product from step D (70 mg, 0.1 mmole) was dissolved in MeOH (2 mL), dioxane (1 mL) and water (1 mL). NaOH aqueous solution (1N, 1 mL) was added. The mixture was stirred for 2 1/2 hours. The water was evaporated with a stream of air to give the product as an oil (70 mg).
NMR (CD_3OD) δ: 3.05-3.95 (multiplet, 7H), 3.00 (singlet, 3H), 4.45 (singlet, 6H), 4.65 (doublet, J = 3.5 Hz, 1H), 5.65 (doublet, J = 3.5 Hz, 1H), 6.20 (singlet, 1H), 6.90-7.20 (multiplet, 3H), 7.82 (doublet, J = 6 Hz, 1H).

Tables 5-12 contain representative protoxins that can be made and used by procedures described heretofore.

TABLE 5

REPRESENTATIVE PROTOXINS

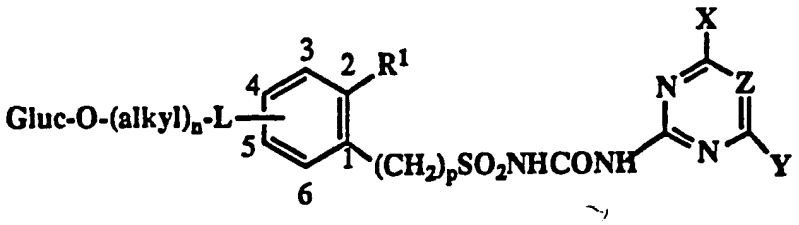


Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
1	H	0	$OCH_2CH_2CH_2$	OCH_3	OCH_3	CH
2	H	0	$OCH_2CH_2CH_2$	CH_3	OCH_3	CH
3	H	0	CH_2CH_2	Cl	OCH_3	CH
4	H	0	CH_2CH_2	CH_3	CH_3	CH
5	H	0	$OCH_2CH_2CH_2$	CH_3	OCH_3	N
6	H	0	$OCH_2CH_2CH_2$	OCH_3	OCH_3	N
7	H	0	OCH_2CH_2	CH_3	OCH_3	CH
8	H	0	OCH_2CH_2	CH_3	CH_3	CH
9	H	0	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
10	H	0	$CO_2CH_2CH_2$	CH_3	OCH_3	N
11	H	0	$CO_2CH_2CH_2CH_2$	OCH_3	CH_3	CH
12	H	0	$CO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
13	H	0	$CH_2CH_2CH_2$	OCH_3	CH_3	N
14	H	0	$CH_2CH_2CH_2$	OCH_3	OCH_3	CH
15	H	0	$OCH(CH_3)CH_2$	Cl	OCH_3	CH
16	H	0	$OCH(CH_3)CH_2$	CH_3	OCH_3	N
17	6-F	0	$C(CH_3)_2CH_2CH_2$	CH_3	OCH_3	CH
18	6-F	0	$C(CH_3)_2CH_2CH_2$	CH_3	OCH_3	N
19	6-Cl	0	$SO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
20	6-Cl	0	$SO_2CH_2CH_2CH_2$	OCH_3	CH_3	CH
21	5-CF_3	0	$CO_2CH_2CH_2$	CH_3	CH_3	CH
22	5-CF_3	0	$CO_2CH_2CH_2$	CH_3	OCH_3	CH

Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
23	H	1	$SCH_2CH_2CH_2$	CH_3	CH_3	CH
24	H	1	$CH(CH_3)$	CH_3	OCH_3	CH
25	H	0	$OCH_2CH_2OCH_2CH_2$	OCH_3	CH_3	N
26	H	0	$C(CH_3)_2CH_2CH_2$	OCH_3	OCH_3	N
27	H	0	$CH_2C(CH_3)_2CH_2$	CH_3	OCH_3	N
28	H	0	CH_2	CH_3	CH_3	CH
29	H	0	$CH(CH_3)CH_2$	CH_3	OCH_3	CH
30	H	0	$CH_2SCH_2CH_2$	CH_3	CH_3	CH
31	H	0	$SO_3CH_2CH_2$	OCH_3	CH_3	N
32	H	0	$CH_2OCH_2CH_2$	OCH_3	CH_3	N
33	H	0	SCH_2CH_2	OCH_3	CH_3	CH
34	H	0	SCH_2CH_2	OCH_3	OCH_3	CH
35	H	0	$SO_2NHCH_2CH_2$	OCH_3	Cl	CH
36	6-Cl	0	$CH_2CH_2CH_2$	CH_3	CH_3	CH
37	6-Cl	0	$CH_2CH_2CH_2$	OCH_3	CH_3	CH
38	6-Cl	0	$CH_2CH_2CH_2$	OCH_3	OCH_3	CH

TABLE 6

REPRESENTATIVE PROTOXINS

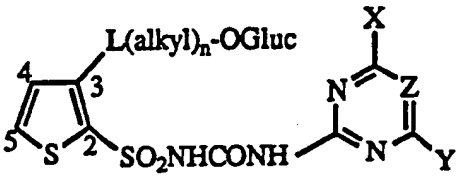


Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
39	CO_2CH_3	0	5-CH_2	CH_3	CH_3	CH
40	CO_2CH_3	1	5-CH_2	OCH_3	CH_3	N
41	CO_2CH_3	0	3-OCH_2CH_2	CH_3	CH_3	CH
42	$CO_2CH_2CH_3$	0	3-CH_2	OCH_3	CH_3	N
43	$CO_2CH_2CH_3$	0	3-CH_2	OCH_3	CH_3	N

Protoxin No.	R^1	p	L(alkyl)$_n$	X	Y	Z
44	$CO_2CH_2CH_3$	0	5-$CH(CH_3)$	CH_3	OCH_3	CH
45	$CO_2CH_2CH_3$	0	3-CH_2CH_2	OCH_3	OCH_3	N
46	Cl	0	6-$CH_2CH_2CH_2$	CH_3	OCH_3	N
47	Cl	0	6-SCH_2CH_2	OCH_3	OCH_3	N
48	Cl	0	5-OCH_2CH_2	CH_3	CH_3	CH
49	Cl	0	6-$OCH_2CH_2CH_2$	OCH_3	CH_3	CH
50	$CH(CH_3)_2$	0	6-$SO_2CH_2CH_2$	CH_3	CH_3	CH
51	$CH(CH_3)_2$	0	6-CH_2	OCH_3	CH_3	CH
52	$CH(CH_3)_2$	0	5-$C(CH_3)_2CH_2CH_2$	OCH_3	OCH_3	N
53	SO_2CH_3	0	3-OCH_2CH_2	OCH_3	OCH_3	CH
54	SO_2CH_3	0	5-$OCH(CH_3)CH(CH_3)$	CH_3	OCH_3	CH
55	SO_2CH_3	0	6-$SO_2CH_2CH_2$	OCH_3	OCH_3	CH
56	$SO_2nC_3H_7$	0	6-OCH_2CH_2	OCH_3	CH_3	N
57	$SO_2nC_3H_7$	0	3-$OCH_2CH(CH_3)$	OCH_3	CH_3	CH
58	$SO_2nC_3H_7$	0	5-$CH(CH_3)CH_2$	CH_3	CH_3	CH
59	Br	0	5-$CO_2CH_2CH_2$	CH_3	CH_3	CH
60	CF_3	0	5-OCH_2CH_2	CH_3	OCH_3	CH
61	NO_2	0	5-CH_2	CH_3	OCH_3	N
62	NO_2	0	3-$OCH_2CH_2CH_2$	OCH_3	OCH_3	N
63	NO_2	0	6-OCH_2CH_2	OCH_3	OCH_3	CH
64	OCH_2CF_3	0	3-$OCH_2CH_2CH_2$	CH_3	CH_3	CH
65	$OCHF_2$	0	5-$C(CH_3)_2CH_2CH_2$	CH_3	CH_3	CH
66	SCF_2CF_2H	0	5-CH_2	CH_3	CH_3	CH
67	Ph	0	5-CH_2CH_2	OCH_3	OCH_3	N
68	$COCH_2CF_3$	0	5-$CH_2CH_2CH_2$	OCH_3	OCH_3	CH
69	$COCH_3$	0	5-$SO_2CH_2CH_2$	OCH_3	OCH_3	N

TABLE 7

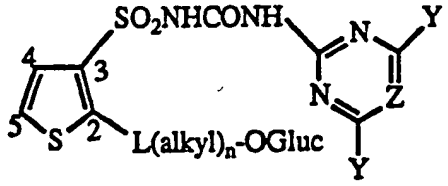
REPRESENTATIVE PROTOXINS



Protoxin No.	L(alkyl)$_n$	X	Y	Z
70	CH_2	CH_3	OCH_3	CH
71	CH_2	OCH_3	OCH_3	N
72	CH_2CH_2	CH_3	CH_3	CH
73	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
74	$CO_2CH_2CH_2$	CH_3	OCH_3	N
75	$CO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
76	$SO_2CH_2CH_2$	CH_3	CH_3	CH
77	$SO_2CH_2CH_2$	CH_3	OCH_3	CH
78	$SO_2CH_2CH_2CH_2$	CH_3	CH_3	N
79	OCH_2CH_2	CH_3	OCH_3	CH

TABLE 8

REPRESENTATIVE PROTOXINS

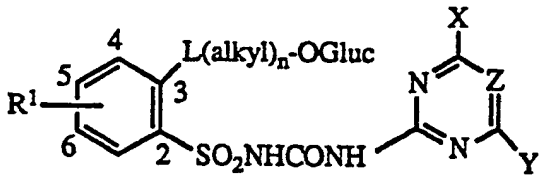


Protoxin No.	L(alkyl)$_n$	X	Y	Z
80	CH_2	CH_3	OCH_3	CH
81	CH_2	OCH_3	OCH_3	N

Protoxin No.	L(alkyl)$_n$	X	Y	Z
82	CH_2CH_2	CH_3	CH_3	CH
83	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
84	$CO_2CH_2CH_2$	CH_3	OCH_3	N
85	$CO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
86	$SO_2CH_2CH_2$	CH_3	CH_3	CH
87	$SO_2CH_2CH_2$	CH_3	OCH_3	CH
88	$SO_2CH_2CH_2CH_2$	CH_3	CH_3	N

TABLE 9

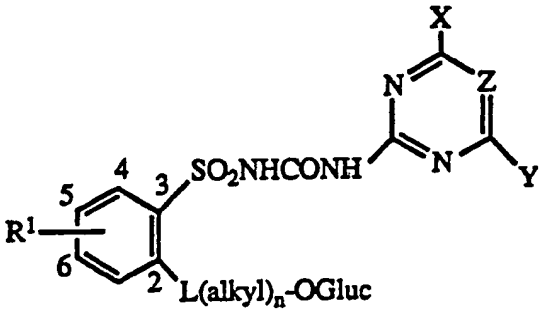
REPRESENTATIVE PROTOXINS



Protoxin No.	R^1	L(alkyl)$_n$	X	Y	Z
89	H	$CO_2CH_2CH_2$	CH_3	CH_3	CH
90	6-OCH_3	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
91	6-CF_3	$CO_2CH_2CH_2$	CH_3	OCH_3	N
92	H	$SO_2CH_2CH_2CH_2$	OCH_3	OCH_3	CH
93	6-CH_3	$SO_2CH_2CH_2CH_2$	CH_3	OCH_3	N
94	6-CF_3	$SO_2CH_2CH_2CH_2$	OCH_3	CH_3	CH

TABLE 10

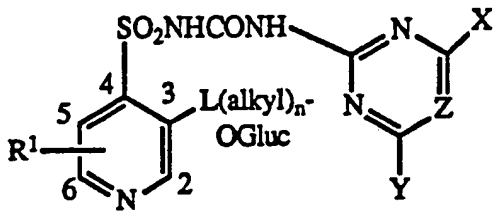
REPRESENTATIVE PROTOXINS



Protoxin No.	R^1	L(alkyl)$_n$	X	Y	Z
95	H	CH_2CH_2	CH_3	OCH_3	CH
96	H	$CH_2CH_2CH_2$	CH_3	OCH_3	N
97	H	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
98	6-CF_3	$SO_2CH_2CH_2CH_2$	CH_3	OCH_3	CH
99	H	SCH_2CH_2	OCH_3	OCH_3	CH
100	6-Cl	OCH_2CH_2	OCH_3	OCH_3	N

TABLE 11

REPRESENTATIVE PROTOXINS

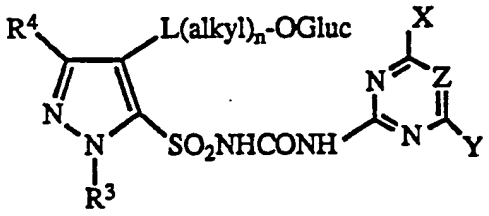


Protoxin No.	R^1	L(alkyl)$_n$	X	Y	Z
101	H	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
102	H	$CO_2CH_2CH_2$	OCH_3	OCH_3	CH

Protoxin No.	R^1	$L(alkyl)_n$	X	Y	Z
103	H	$N(CH_3)-CH_2CH_2$	OCH_3	OCH_3	CH
104	H	$N(CH_3)-CH_2CH_2$	OCH_3	CH_3	CH
105	H	$CO_2CH_2CH_2$	CH_3	OCH_3	N
106	H	$CO_2CH_2CH_2CH_2$	OCH_3	OCH_3	N
107	H	$SO_2CH_2CH_2CH_2$	CH_3	CH_3	CH
108	2-CF_3	SCH_2CH_2	OCH_3	CH_3	CH
109	6-Cl	$CO_2CH_2CH_2$	OCH_3	CH_3	CH
110	H	$SO_2CH_2CH_2$	OCH_3	CH_3	CH

TABLE 12

REPRESENTATIVE PROTOXINS



Protoxin No.	R^3	R^4	$L(alkyl)_n$	X	Y	Z
111	CH_3	H	CH_2CH_2	CH_3	OCH_3	CH
112	CH_3	H	CH_2CH_2	CH_3	OCH_3	N
113	CH_3	H	CH_2CH_2	CH_3	CH_3	CH
114	CH_3	H	CH_2CH_2	OCH_3	OCH_3	N
115	CH_3	H	CH_2CH_2	OCH_3	OCH_3	CH
116	CH_3	H	$CO_2CH_2CH_2$	CH_3	OCH_3	CH
117	CH_3	H	$CO_2CH_2CH_2$	CH_3	OCH_3	N
118	CH_3	H	$CO_2CH_2CH_2$	OCH_3	OCH_3	CH

EXAMPLE 12

1-(6-Hydroxy-3-pyradazinyl)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronic acid, methyl ester

A solution of maleic hydrazide (0.28 g, 2.5 mmole) in anhydrous dimethylformamide (10 mL) under nitrogen atmosphere and stirring, was warmed to 110°C. A solution of methyl 2,3,4-tri-O-acetyl-1-bromo-α-D-glucuronate (2 g, 5 mmole) in anhydrous dimethylformamide (6 mL) was added in six portions, with a time interval of five minutes between each addition. After the addition, the reaction solution was further stirred at 110°C for 20 min. The reaction solution was then cooled to room temperature, poured into water (100 mL), and extracted with ethyl acetate (3 X 75 mL). The combined ethyl acetate extracts were washed with brine, dried over anhydrous magnesium sulfate, and filtered. The filtrate was blown with a stream of air for 18 h. The residue was taken up in methylene chloride. The undissolved solid was filtered and the filtrate was concentrated under reduced pressure to an oil which was chromatographed on a silica gel column (EtOAc) to give the title product as a yellow solid (0.36 g, 34% yield, m.p. 162-165°C). Chemical Inonization (CI) mass spectrum: 303 ($M^+ + 1$).

EXAMPLE 13

1-(6-Hydroxy-3-pyradazinyl)-β-D-glucopyranosiduronic acid, methyl ester

1-(6-Hydroxy-3-pyradazinyl)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronic acid, methyl ester (2.0 g, 4.7 mmole) was dissolved in a mixture of methanol (40 mL) and methylene chloride (20 mL) at room temperature. A solution of sodium methoxide in methanol (25% by weight, 20 drops) was added. The reaction was monitored by thin layer chromatography (methanol/methylene chloride = 1/4). When no more of the starting sugar was present,

resin (Amberlyst 15 ion exchange r sin, strongly acidic, pre-washed with water until the washing was pH neutral) was added to bring the pH of the reaction solution to neutrality. The resin was then filtered, and the filtrate was blown with a stream of air overnight to a residue which was chromatographed on silica gel column (methanol/methylene chloride = 1/4) to give the product (0.5 gm, 35%, m.p. 180-183°C). Fast-atom bombardment (FAB) mass spectrum: 303 ($M^+ + 1$).

EXAMPLE 14

Enzymatic Synthesis

Some aglycones that are difficult to make into glucuronides by conventional chemical synthesis means can be glucuronidated by enzymatic procedures. Glucuronidation is a major means of detoxification and elimination of xenobiotics in mammals. The glucuronidation enzyme, UDP-glucuronosyl transferase, is present in many organs, with high levels in the liver. The enzyme UDP-glucuronosyl transferase conjugates a variety of aglycones onto UDP-glucuronic acid to form β-O-glucuronides. Immobilized rabbit liver microsomal UDP-glucuronosyl transferase has been shown to perform conjugation *in vitro*. Some glucuronide protoxins were prepared this way.

Liver microsomal UDP-glucuronosyl transferase was extracted from phenobarbitol-treated New Zealand White rabbits and immobilized as enzyme beads as described by Lehman et al., *Drug Metab. Disp.*, 9:15-18 (1981). For most conjugation reactions, half of the reaction volume was made of the suspended enzyme beads in 100 mM phosphate buffer at pH 7.4. The concentrations of UDP-glucuronic acid (ammonium salt) and the aglycone in the reaction mix were 10 mM and 2 mM, respectively. Magnesium chloride at 4 mM was used to facilitate the reaction. If the aglycone was difficult to solubilize

in aqueous solution, up to 5% ethanol was used to achi ve 2 mM concentration. Due to the relatively high β-glucuronidase (GUS) activity in the crude rabbit liver microsome enzyme preparation, 150 mM glucaro-1,4-lactone was included in the reaction to suppress GUS activity. If the reaction was run for more than one day, UDP-glucuronic acid was replenished daily. The reaction vessel was shaken gently at 37°C for the entire period.

Progress of the enzyme conjugation reaction was monitored by high pressure liquid chromatography (HPLC) analysis of small aliquots of the reaction mixture. For most analyses a C_{18} reverse phase column, 25 cm long, 4.6 mm inner diameter, was used. The mobile phase was a mixture of water and acetonitrile (ACN) with 0.1% formic acid. Usually the mobile phase gradient was from 5% ACN to 80% ACN in 25 minutes, at a 1.4 mL per minute flow rate. The column temperature was 35°C. After sufficient glucuronide conjugate was synthesized, as judged by the HPLC analysis, the reaction was terminated. The enzyme beads were filtered out, and the glucuronide was isolated from the reaction solution by the following procedure: adjusted pH to 2.5/partition 3x with methylene chloride/ obtained a methylene chloride phase (contains aglycone) and an aqueous phase/partition 3x with n-butanol/obtain a butanol phase (glucuronide) and an aqueous phase.

The butanol fraction which contains the glucuronide, a small amount of the aglycone and UDP-glucuronic acid was evaporated to dryness in a rotary evaporator. It was then further purified by a preparative HPLC. A one-inch diameter C_{18} reverse phase column, 25 cm in length, was used. An isocratic mobile phase with 15 to 35% ACN in water, 0.1% formic acid was used at a 15 mL per minute flow rate. The column was kept at room temperature. The glucuronide peak was

collected by hand. Th pH of the glucuronide fraction was quickly adjusted to neutrality. The ACN in the fraction was evaporated by placing under a nitrogen gas stream in a hood. The remaining aqueous fraction was adjusted to pH 2.5 and extracted three times with butanol. This butanol fraction was taken to dryness in a rotary evaporator with gentle heating. The resulting pure glucuronide was stored in a -20°C freezer.

Glucuronides of the following compounds (herbicidal sulfonylureas with a free hydroxyl group) were prepared by enzymatic techniques:

Cmpd. No.	
36	2-chloro-N-[[(4,6-dimethyl-2-pyrimidinyl)amino]-carbonyl]-6-(3-hydroxypropyl)benzenesulfonamide,
37	2-chloro-6-(3-hydroxypropyl)-N-[[(4-methoxy-6-methyl-2-pyrimidinyl)amino]carbonyl]benzene-sulfonamide,
38	2-chloro-N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]-carbonyl]-6-(3-hydroxypropyl)benzenesulfonamide,
119	2-[(2-hydroxyethyl)thio[-N-[[(4-methoxy-6-methyl-2-pyrimidinyl)amino]carbonyl]benzenesulfonamide,
120	N-[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]-carbonyl]-N'-(2-hydroxyethyl)-1,2-benzenedi-sulfonamide,
121	N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-6-(2-(N'-(2-hydroxyethyl)-N'-methyl)amino)-2-pyridinesulfonamide,
122	6-[(2-hydroxyethyl)methylamino]-N-[[(4-methoxy-6-methyl-2-pyrimidinyl)amino]carbonyl]-2-pyridinesulfonamide, and
123	N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-2-[(2-hydroxyethyl)thio]benzenesulfonamide.

The enzymatic conjugation procedures were as described above. In all reactions, the glucuronides showed a shorter retention time (about 2-2.5 minutes) than their aglycones in the HPLC chromatogram. The UV absorption spectra of all glucuronides were identical or nearly identical to those of their respective aglycones. When the aliquot of the reaction was incubated with the GUS enzyme, the glucuronide peak disappeared and the aglycone peak became larger. After purification, glucuronides were again analyzed by HPLC to check sample purity. The chemical identities of these glucuronides were further confirmed by LC/MS/FAB analysis.

The substrate qualities of the glucuronides produced were assayed by incubation with pure GUS enzyme. The same concentration of glucuronide (0.5 mM) was used in 2 mL, with 25 units (1 unit will liberate 1.0 µg of phenolphthalein from phenolphthalein glucuronide per hour at 37°C) of GUS. At different time points the reaction was stopped by pipetting in sodium carbonate solution to raise the pH to 11.8. The resulting samples were analyzed by HPLC. The peak area ratio of the glucuronide over the aglycone was used to indicate the degree of hydrolysis. It is clear that different glucuronides were hydrolyzed by GUS at different rates.

EXAMPLE 15

Demonstration of in vivo β-glucuronidase activity in TA29-GUS tobacco anthers

Although the GUS gene has been widely used in plant molecular biology research, the demonstration of β-glucuronidase enzyme activity is usually done *in vitro*. The transgenic plant material harbouring the GUS gene product, β-glucuronidase, is disrupted for color staining or other assays for the enzyme activity. It is generally assumed that the activity of β-glucuronidase

detected in vitro is well correlated with the in vivo activity.

To demonstrate β-glucuronidas activity in vivo, 4-methylumbelliferone glucuronide (MUG) was fed to tobacco plants described in Examples 3 and 4, which exhibited anther-specific GUS expression by in vitro assays, using the cotton wick method as described below. Following different times after the feeding, anthers of young flowers were collected and assayed for the presence of MUG, 4-methylumbelliferone (MU) and MU-glucoside. The phosphate buffer extract of anthers was divided into three aliquots. One aliquot was assayed for MU by fluorescence measurement. The second aliquot was treated with β-glucosidase, followed by MU measurement. This value represents the MU, generated from MUG by the GUS enzyme, which was reconjugated into a beta-glucoside by the plant. The third aliquot was treated with the GUS enzyme, followed by MU measurement. This value represents the unhydrolyzed MUG in anthers. The MU values of the first and second aliquots together represent the amount of MUG hydrolysis by GUS. It is clear that MUG, after being translocated into the anther, was abundantly hydrolyzed by the GUS enzyme into MU as described hereafter. Under similar conditions MUG was minimally hydrolyzed in a non-transgenic plant.

EXAMPLE 16

Testing of protoxin/proCHA on transgenic plants producing male organ-specific exogenous enzyme

The protoxin solution was locally applied to the inflorescence stalk of the transgenic plant through a fine cotton wick. The wick, pulled through the stalk by a sewing needle, was usually embedded at a point about one to two centimeters from flower clusters. The concentration of protoxin varied depending on the toxicity of the aglycone and the size of the

inflor scence. One properly embedded wick can deliver up to 1.5 mL of solution per day into the inflor scence stalk, and it can function well for over a week.

Freshly shed pollen from treated flowers was assayed for germinability in Brewbaker and Kwak medium (15% sucrose, 300 ppm calcium nitrate, 100 ppm boric acid). To test the degree of male sterility more definitively, the same treated pollen was also used to pollinate the stigma of untreated flowers. If a fruit resulted from such pollination, it indicated that the treatment did not cause complete male sterility.

Glucuronides of 2-[(2-hydroxyethyl)-thio]-N-[[(4-methoxy-6-methyl-2-pyrimidinyl)amino]-carbonyl]-benzenesulfonamide and N-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-6-[(2-hydroxyethyl)-methylamino]-2-pyridinesulfonamide were fed into infloresence stalks of tobacco plants described in Examples 3 and 4 exhibiting anther-specific GUS activity by the cotton wick method. At the beginning of the feeding, the infloresence was very young with all of its flower buds entirely enclosed within their sepals. The concentrations of the glucuronides were between 1 - 3 parts per million (ppm), depending on the size of the infloresence. The chemical solution uptake rate was about 0.5 to 1.0 mL per day. More solution was added to the small vial in which the cotton wick was bathed. The solution addition was calibrated according to the growth of flowers on the inflorescence. All treated infloresences went on to produce flowers. Freshly shed pollen from treated flowers was used to pollinate untreated flowers. A small fraction of the pollen was assayed for germinability in Brewbaker and Kwak medium (supra). On the same day, treated flowers were pollinated with pollen from untreated tobacco plants to check their female fertility.

The female fertility of all treated flowers was completely normal. A few days after receiving untreated pollen, nice plump seed pods began to develop. However, treated flowers failed to produce functional pollen. Their freshly shed pollen grains showed no germination, or much reduced germination, in Brewbaker and Kwak medium. When such pollen was used to pollinate the untreated flowers, much reduced rates of seed pod formation were observed. The degree of male sterility achieved by this localized chemical application via a cotton wick can be variable. The outcome of the test is influenced by a variety of factors, such as environmental conditions, developmental stages of test plant, position and size of the treated inflorescence.

EXAMPLE 17

Transgenic tobacco plants as described in Example 4 were sprayed with the methyl ester of maleic hydrazide glucuronide. The pod-forming ability of said treated plants was adversely affected versus control plants.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: HSU, FRANCIS C.
ODELL, JOAN T.

(ii) TITLE OF INVENTION: COMPOUNDS AND CONSTRUCTS FOR PRODUCING MALE STERILE PLANTS

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:

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(E) COUNTRY: USA

(F) ZIP: 19898

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/578,360

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(A) TELEPHONE: 302-992-4926

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(C) TELEX: 835420

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 549 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCTAGCTAA GTATAACTGG ATAATTTGCA TTAACAGATT GAATATAGTG CCAAACAAGA 60

AGGGACAATT GACTTGTCAC TTTATGAAAG ATGATTCAAA CATGATTTTT TATGTACTAA 120

TATATACATC CTACTCGAAT TAAAGCGACA TAGGCTCGAA GTATGCACAT TTAGCAATGT 180

AAATTAAATC AGTTTTTGAA TCAAGCTAAA AGCAGACTTG CATAAGGTGG GTGGCTGGAC 240

TAGAATAAAC ATCTTCTCTA GCACAGCTTC ATAATGTAAT TTCCATAACT GAAATCAGGG 300

TGAGACAAAA TTTTGGTACT TTTTCCTCAC ACTAAGTCCA TGTTTGCAAC AAATTAATAC 360

ATGAAACCTT AATGTTACCC TCAGATTAGC CTGCTACTCC CCATTTTCCT CGAAATGCTC 420

CAACAAAAGT TAGTTTTGCA AGTTGTTGTG TATGTCTTGT GCTCTATATA TGCCCTTGTG 480

GTGCAAGTGT AACAGTACAA CATCATCACT CAAATCAAAG TTTTTACTTA AAGAAATTAG 540

CTACCATGG 549

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 337 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CACACACAAA AACTAAGTAA AAAGAAGAAA AAGCCTTTCA GGTCTCAACA ATGGAGATGA 60

AGAAGATCGC TTGCGGTGTG CTTTTTGTTG CTGCCTCCAT GACTGCTGTC ATGGCTATTG 120

AGGAAGCTGG AGCTCCGGCA CCAGGACCCG CATCCGCCGC CCTCGGTTGC ATTGCCGGCT 180

CTTGGCTCGT TGGTTGGTGC TTCGCTTGTG TCCCTATTCA GCTACTACTT GCACTAAGCT 240

ATGTCTTCAT TATGTAATAA TACTATTTCT GTGATCTTTT TCATTTCCTA CCTATTTCCA 300

TTTCACTTGT TTATTAATAA AACTGATAAA ATATTTC 337

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 554 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCACATGCC TCCTCATCCT TACGATCTAC ATTGCAGCTC CAACAGAGTC ACACATAACG 60

TGTGGGACAG TGACAAGCAC AATGACACAG TGCATCAGCT ACTTGACCAA CGGTGGTCCA 120

TTGCCGTCAA GCTGCTGTGT GGCAGTCAAA TCATTGAACC AATGGCTCAG ACCCACCAGA 180

TCGCCGACAA GTAGTGTAGT CCTTAAATCA GCCGGTAAGG AAATTAAAGG CCTCAATATC 240

GACCTTGTGG CCGGCACTCC CTACCACTTG TGGTGTTTCA CTTTCATACC CCATTGGTTT 300

CAACACCAAT TGTGACAGTA TATCGATTGC CGTGTGAAGG AGACTAGAGA TGTACGAACG 360

AATAATCAAA GTTGGCCCGA CTTTAACCTA AACAATACTT CTGCTATTTT CTATTTTATG 420

ACTTGAGTTT CTTATTATGT GATCCATTTC ATATGGTAAT AAGTAATAAC GATAAAGGAG 480

CTGTCTCTCC TTTTCGACGC TTTGATGTAT GTACACGTAA CATACCATCA GGAATAACAC 540

TTTGTCTTTC TCTT 554

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 406 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACAACCTT CAGGAATATT AGAGGAACAT CAGAGAACAA GGACGCAGTG AAACTATTGT 60

GCAGCAAGGG ACATCCATGT GAGAACGTTG AGATTGGAGA CATTAACATT GAGTACACAG 120

GACCTGACGG TCCACCCACT TTCGAGTGCA CAAACGTCAC ACCTAAGCTT GTGGGAGCCC 180

AGAACCCAAA GGCTTGCGTT GGACCTGTGG TCAAGGCTCC TGGCAAAGCG TAAAATGTTG 240

AAGCTCAGAT CAACAAACTA GGGCTTTCAC ATCCAATTTT GTTTTTCCTT TTCTCTAACC 300

CTTTTTCAAG TCACATATGG GTGAGTTTTA GAAAACTGTA ACCAAAAAAA TGTATATTCG 360

ATGCCAACAC ATATGTGTGA GTGCATAATG ATGTAATTAA TAAAAC 406

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 971 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGTGACAAGC ACAATGACAC AGTGCATCAG CTACTTGACC AACGGTAGGT CCATTGCCGT 60

CAAGCTGCTG TGTGGCAGTC AAATCATTGA ACCAAATGGC TCAGACCACA CCAGATCGCC 120

GACAAGTATG TGAGTGCCTT AAATCAGCCG GTAAGGAAAT TAAAGGCCTC AATATCGACC 180

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GGAGGAGGCC AACCCTCGGG TGATTGTTGT GCAAAGCTGA AGGAGCAGCA GCCATGCTTA 720

TGTGGATTTG CTAAGAACCC TGCGTTTGCT CAGTACATTA GCTCTCCGAA CTCTCGACAA 780

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CAAACAAGTA ACCGTTTGGT TAAGTAATCG TATACGGTTC ATCAAAGAAG TTTCTTATTT 960

AAAAAAAAAA A 971

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 747 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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AGGGAGCCGG GTGCTGCTCA CCACCTCCGA GACTATGGTT CTTGGGTTCC GTCCCCCCAA 120

CAAAGCTCGC CCTTACGACT TAGTCGGGGC TGCTCTCTTT GGAGACGGAG CAGCTGCCCT 180

GATCATCGGA GCAGACCCTA CAGAGTCAGA AAGTCCCTTC ATGGAGCTTC ACTATGCGCT 240

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CAGAGAAGTT GGAATGCAGC AGACAGGCGT TGGTGGATTA TGGGAACGCA AGCAGCAACA 540

CCATCTTCTA CATAATGGAC AAAGTAAGAG ATGAGCTTGA GAAGAAAGGC AGAAGTGGAG 600

AAGAGTGGGG TCTGGGTTTA GCTTCGGACC GGGAATTACA TTCGAGGGAT TTCTCATGAG 660

GAGCCTCTAA ATGTGGCTAC TGGCTATGGA CCCGACATAT GTGATTAACT AGAAGTAAGA 720

ACAAATAAAT AAAACAGTCT CTTTTGC 747

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 871 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATTCTCGTG CCAGCAACTA TGCCACCACT TTGTTAACTA CGGGTTTCAC GACCTCCGGG 60

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GCATATGAAT CTACATGGAA ATATGTAAAT TTAATACATA TGCTTGGCTA TAATATAATT 240

ATTTACATCA ACGAAGATTC AGGTACATAG TTTCGTAGCT ACAATATATA NNNNNNNNCA 300

CATCTTCCGG CTTACCAAGT AATTAACAAA CCTGCGTTGA TGACACACTT AGATTAACTA 360

TTCTAAAACA CATTAGACGA TTAGTGTCGA GTGTTCAGGA GGCTGATTTC GCTATATTAG 420

TTTCTGAACA TAAAATAATT TATTTCTAAT TTTAAATATA TATCGTGTGT GCAGGCGTCT 480

TAGGAAGAAG GGGAAAGGAA CGCCAAAAAT TCCGGGATTG GCCCCGGGGG CTCCAGATTC 540

TGATCCAGTG TCTGGAGGAT AAACGGAGTA GGCTTTCGTA GTATGAGATA ATCTATGGAG 600

GAATTTAATG AGAATTGCTT GGGGTTCCTT AAATAAAACC TAAAATTATC CAGTGTCTTT 660

AACGTAAGAC TGATTACGCG CGGATGAAAC TAATGTTTCN NNNNNNNGGA TGAAACATTA 720

GTTATATACC ATTTTCAAAG AATATCGTGT TACGAATATA ATAAAGTACT CGATCATGTT 780

GTATATATTA AAGAATAAGT GATATCACTG AGTTACATAG ATGTATATTC AAGTTACATT 840

ATTGATTCAA TGAATTATTC GACTTTGAAA C 871

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGAAATTAGC TACCATGGTA GCTCCAAAAT 30

CLAIMS

What is claimed is:

1. In a method for inducing sterility in the male organ of a plant that comprises the steps:

(i) transforming the plant with a DNA construct that combines a male-organ specific promoter with the coding region for β-glucuronidase; and

(ii) contacting the transformed plant with a glucuronic acid-containing protoxin to release a toxin which renders the male organ sterile;

the improvement that comprises:

(a) employing, in step (i), a promoter selected from the group $TA29_{1500}$, $TA29_{500}$ (SEQ ID NO:1), p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7); and

(b) employing, in step (ii), a protoxin that comprises a toxin conjugated through a non-acyl, non-phosphoryl hydroxyl residue to glucuronic acid.

2. A method according to Claim 1 comprising transforming, in step (i), an agricultural or horticultural plant.

3. A method according to Claim 2 wherein the plant is selected from the group corn, rice, orchardgrass, soybean, cotton, Brassica, pea, pepper, potato, sugarbeet, alfalfa, sunflower, tobacco, flax, tomato, lettuce, celery, carrot, eggplant, apple, melon, petunia, periwinkle, poplar and walnut.

4. A DNA construct combining a promoter selected from the group $TA29_{1500}$, $TA29_{500}$ (SEQ ID NO:1), and

promot rs from genes represent d by p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7) with the coding region for β-glucuronidase in the PZS96 Agrobacterium binary vector.

5. A protoxin comprising a sulfonylurea compound or maleic hydrazide conjugated to glucuronic acid.

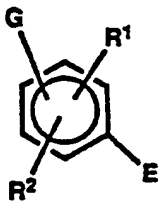
6. A protoxin according to Claim 5 wherein the conjugate is with a sulfonylurea compound.

7. A protoxin according to Claim 5 wherein the cojugate is with maleic hydrazide.

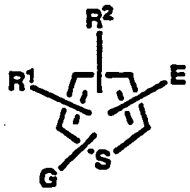
8. A protoxin according to Claim 6 comprising a sulfonylurea of Formula I including agriculturally suitable isomers, salts and derivatives thereof:

I

wherein J is



J-1



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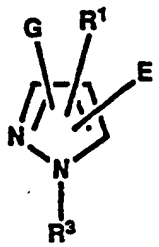

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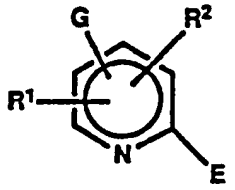
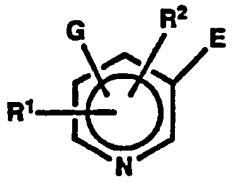


J-4



J-5

G is H or gluc-O(alkyl)$_n$L;

n is 0 or 1;

alkyl is 1 to 3 carbon atoms optionally substituted with one or two groups selected from halogen, methyl, methoxy or methylthio;

L is O; S(O)$_m$; NR^5; SO_2NR^4; CO_2; CH_2O; or a direct bond;

m is 0-3;

W is O or S;

R, R^4 and R^5 are independently H or CH_3;

E is a single bond or CH_2;

R^1 is H, C_1 to C_3 alkyl, C_1 to C_3 haloalkyl, halogen, nitro, C_1 to C_3 alkoxy, $SO_2NR^aR^b$, $CONR^aR^b$, C_1 to C_3 alkylthio, C_1 to C_3 alkylsulfinyl, C_1 to C_3 alkylsulfonyl, CH_2CN, CN, CO_2R^c, C_1 to C_3 haloalkoxy, C_1 to C_3 haloalkylthio, C_2 to C_4 alkoxyalkyl, C_3 to C_4 alkoxyalkoxy, C_2 to C_4 alkylthioalkyl, CH_2N_3, NR^dR^e, or Q;

R^2 is H, C_1 to C_3 alkyl, C_1 to C_3 haloalkyl, halogen, nitro, C_1 to C_3 alkoxy, C_1 to C_3 alkylthio, CN, C_1 to C_3 haloalkoxy, or C_2 to C_4 alkoxyalkyl;

R^a is H, C_1 to C_4 alkyl, C_2 to C_3 cyanoalkyl, methoxy or ethoxy;

R^b is H, C_1 to C_4 alkyl or C_3 to C_4 alkenyl; or

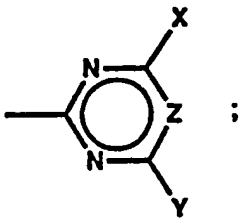
R^a and R^b can be taken together as $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$ or $-CH_2CH_2OCH_2CH_2-$;

R^c is C_1 to C_4 alkyl, C_3 to C_4 alkenyl, C_3 to C_4 alkynyl, C_2 to C_4 haloalkyl, C_2 to C_3 cyanoalkyl, C_5 to C_6 cycloalkyl, C_4 to C_7 cycloalkylalkyl or C_2 to C_4 alkoxyalkyl;

R^d and R^e are independently H or C_1 to C_2 alkyl;

Q is a saturated or partially saturated 5- or 6-membered carbocyclic ring, containing either one or two carbonyl groups, or a saturated or unsaturated 5- or 6-membered heterocyclic ring, containing 1 to 5 atoms of carbon and 1 to 4 heteroatoms selected from the group consisting of 0 to 2 oxygen, 0 to 2 sulfur and 0 to 4 nitrogen, wherein sulfur can take the form of S, SO or SO_2, and containing 0 to 2 carbonyl groups; Q can further be optionally substituted with 1 to 2 substituent groups; substituents on carbon can be selected from the group consisting of halogen, C_1 to C_4 alkyl, C_1 to C_4 haloalkyl, CH_2(C_2 to C_3 alkenyl), CH_2(C_2 to C_3 alkynyl), C_2 to C_4 alkoxycarbonyl, CN, OH, C_1 to C_3 alkoxy, C_1 to C_3 alkylthio, C_1 to C_3 alkylsulfinyl, C_1 to C_3 alkylsulfonyl or C_2 to C_4 alkylcarbonyl; substituents on nitrogen can be selected from the group consisting of C_1 to C_4 alkyl, C_1 to C_4 haloalkyl, CH_2(C_2 to C_3 alkenyl), CH_2(C_2 to C_3 alkynyl), C_2 to C_4 alkoxycarbonyl or C_2 to C_4 alkylcarbonyl;

A is



;

X is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkoxy, C_1 to C_4 haloalkyl, halogen, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to C_3 alkylamino, di(C_1 to C_3 alkyl)amino, C_3 to C_5 cycloalkyl, C_1 to C_4 alkyl substituted with -O-gluc, C_2 to C_4 alkoxyalkyl substituted with -O-gluc, or C_1 to C_4 alkoxy substituted with -O-gluc;

Y is H, C_1 to C_4 alkyl, C_1 to C_4 alkoxy, C_1 to C_4 haloalkoxy, C_2 to C_5 alkoxyalkyl, C_2 to C_5 alkoxyalkoxy, amino, C_1 to C_3 alkylamino or di(C_1 to C_3 alkyl)amino;

R^3 is H or C_1 to C_3 alkyl;

Z is CH or N;

E^1 is a direct bond or CH_2;

gluc is β-D-glucuronic acid;

provided that:

(i) when G is H then X is C_1 to C_4 alkyl substituted with gluc, or C_1 to C_4 alkoxy substituted with gluc;

(ii) when L is not a direct bond, then n is not zero and alkyl comprises at least two carbon atoms which can be substituted as described.

9. A transgenic plant containing a DNA construct of Claim 4.

10. A transgenic plant according to Claim 9 that has been contacted with a protoxin comprising a sulfonylur a compound or maleic hydrazide conjugated to glucuronic acid.

11. Hybrid seed from a plant according to any one of Claim 9 or 10.

12. A male-organ specific promoter, $TA29_{500}$ (SEQ ID NO:1) comprising a nucleic acid fragment derived from the TA29 gene that extends from the Eco RV restriction site, which is about 500 base pairs 5' to the transcription site and extending to the translation initiation ATG.

13. A male-organ specific promoter from genes represented by p73 (SEQ ID NO:2), p112 (SEQ ID NO:3), p54 (SEQ ID NO:4), p42W (SEQ ID NO:5), p42 (SEQ ID NO:6) and p158 (SEQ ID NO:7).

14. A method for planting crops preparatory to field hybridization comprising the steps:

(i) planting crop A^1 and crop A^2 in sufficient proximity that crop A^1 can be fertilized by crop A^2;

(ii) inducing male sterility in A^1, before fertilization, by a method according to Claim 1;

(iii) fertilizing A^1 with the pollen from A^2;

wherein A^1 comprises a plant lacking a desirable heritable trait that is present in A^2 or will be present in the hybrid progeny.

15. A method according to Claim 14 comprising the additional step of harvesting hybrid seed from crop A^1.

FIG. 1A

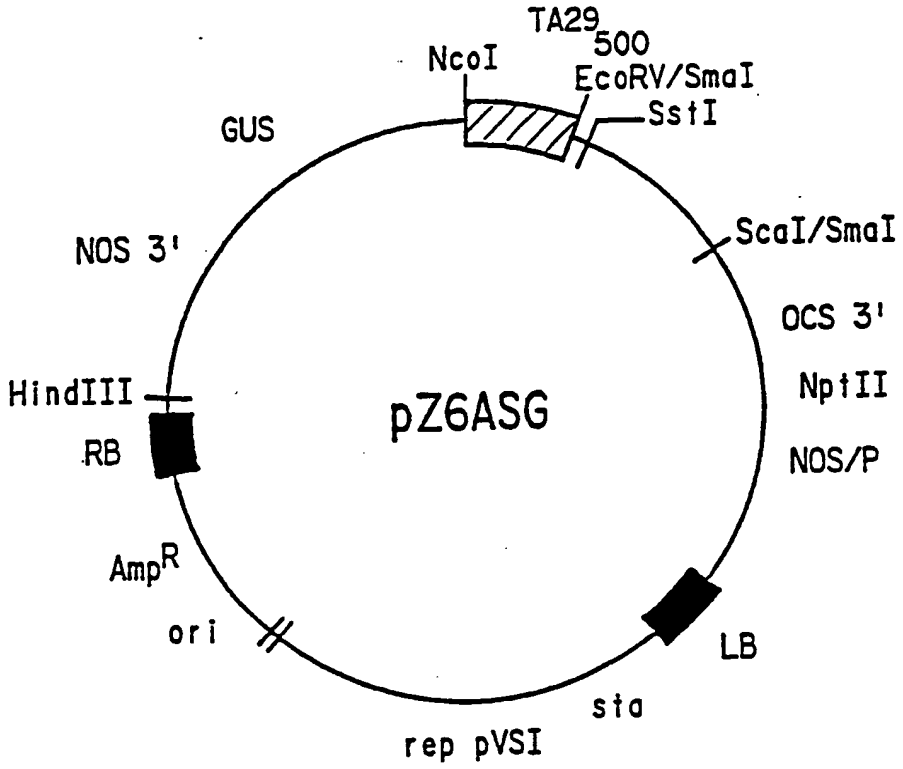

TA29
500
NcoI
EcoRV/SmaI
SstI
GUS
ScaI/SmaI
NOS 3'
OCS 3'
HindIII
NptII
pZ6ASG
RB
NOS/P
Amp^R
ori
LB
sta
rep pVSI

FIG. 1B

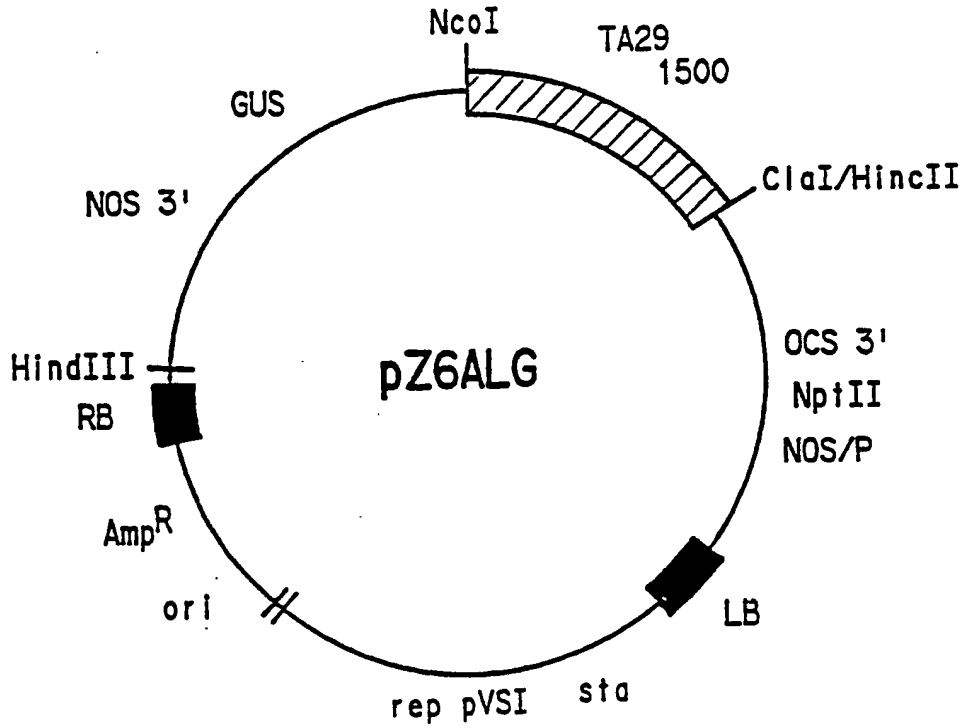

NcoI
TA29
1500
GUS
ClaI/HincII
NOS 3'
OCS 3'
HindIII
NptII
pZ6ALG
RB
NOS/P
Amp^R
ori
LB
rep pVSI
sta

FIG.2A

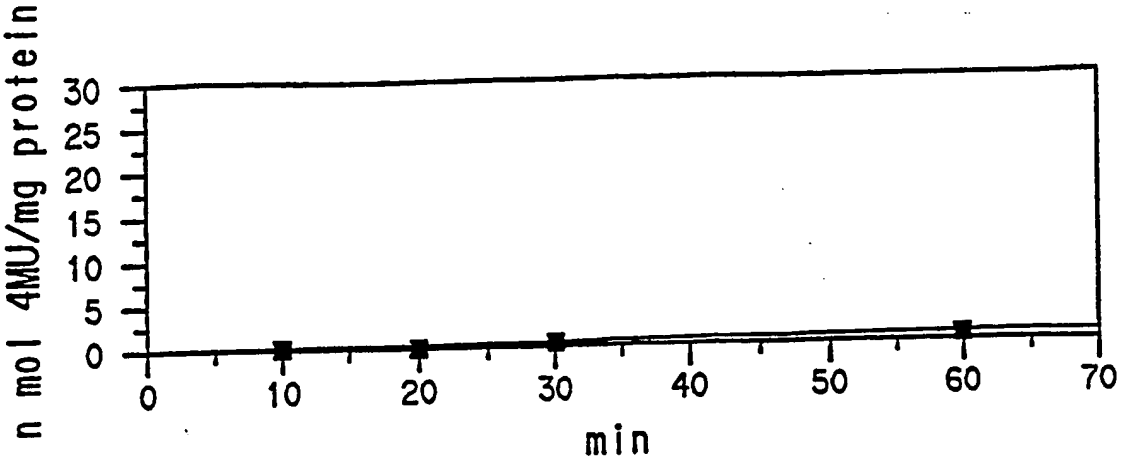


FIG.2B

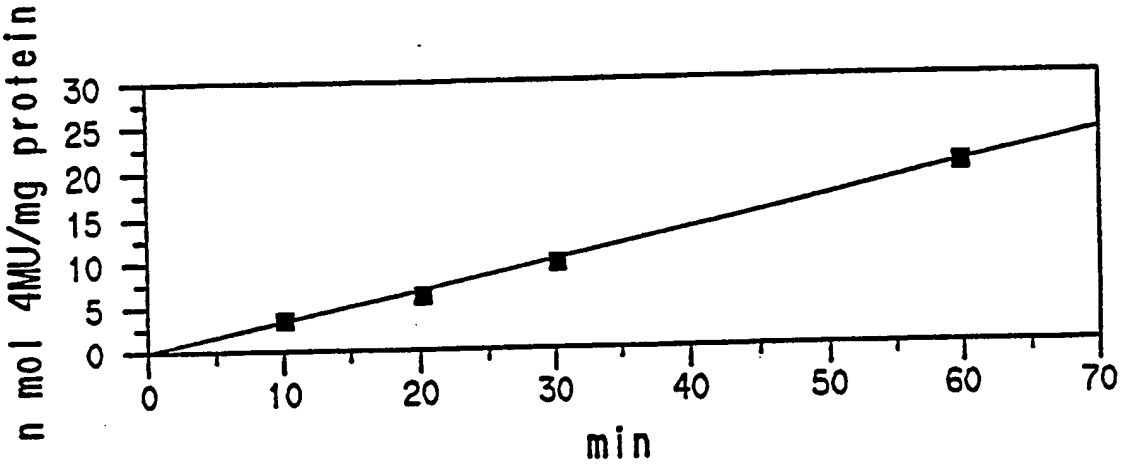
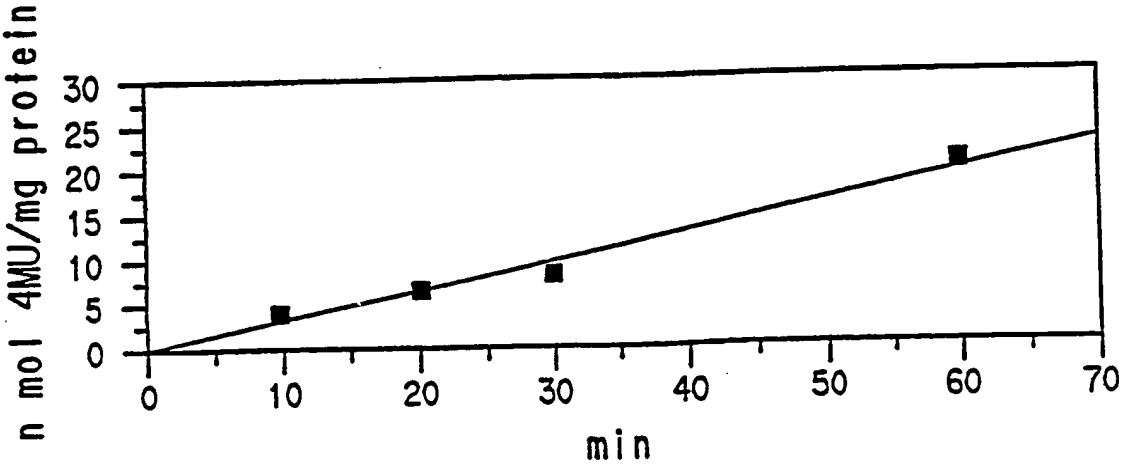


FIG.2C



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/0623

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/29; C12N15/56; C07H15/203
C07H15/26; C07H17/02; A01H1/02; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07H ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	EP,A,0 344 029 (PLANT GENETIC SYSTEMS) 29 November 1989 see figure 5; examples 3,5,6	4,9 1-3,11, 14
Y	WO,A,9 008 828 (PALADIN HYBRIDS) 9 August 1990 see page 49, line 32 - page 50, line 3 see page 109, line 20 - line 35; figure 15	1-3,11, 14
Y	NATURE. vol. 342, 14 December 1989, LONDON GB pages 837 - 838; JEFFERSON, R. A.: 'The GUS reporter gene system' see page 838, left column	1-3,11, 14

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 JANUARY 1992	14 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 103, 1985, Columbus, Ohio, US; abstract no. 16329, OIDA, T., ET AL: 'The metabolism of gliclazide in man' page 8 ; see abstract & XENOBIOTICA vol. 15, no. 1, 1985, pages 87 - 96; ---	5,6
X	CHEMICAL ABSTRACTS, vol. 88, 1978, Columbus, Ohio, US; abstract no. 31950, GAFITEANU, E., ET AL.: 'Elimination of p-bromohydrazino- and [p-bromophenyl]hydrazinopyridazine from the organism' page 21 ; see abstract & REV. MED.-CHIR. vol. 78, no. 3, 1974, pages 703 - 706; ---	5,7
P,X	THE PLANT CELL. vol. 2, no. 12, December 1990, ROCKVILLE, MD, USA. pages 1201 - 1224; KOLTUNOW, A. M.,ET AL.: 'Different temporal and spatial gene expression patterns occur during anther development' see figure 12 ---	4,12
A	EP,A,0 329 308 (PALADIN HYBRIDS) 23 August 1989 see the whole document ---	4,13
A	WO,A,9 008 830 (ICI) 9 August 1990 see examples 9,10 ---	4

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9106234 SA 52415

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent ffice EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/01/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0344029	29-11-89	AU-A-	3537189	24-11-89
		WO-A-	8910396	02-11-89
		JP-T-	2503988	22-11-90
WO-A-9008828	09-08-90	AU-A-	5037290	24-08-90
		EP-A-	0456706	21-11-91
EP-A-0329308	23-08-89	AU-A-	2963289	03-08-89
WO-A-9008830	09-08-90	AU-A-	4945690	24-08-90
		EP-A-	0455665	13-11-91